University of Bath

PHD

Investigation into microbiological and biochemical factors that influence the maturation flavour of strong cider

Swaffield, Claire Helen

Award date:
1996

Awarding institution:
University of Bath

Link to publication

Alternative formats
If you require this document in an alternative format, please contact:
openaccess@bath.ac.uk

Copyright of this thesis rests with the author. Access is subject to the above licence, if given. If no licence is specified above, original content in this thesis is licensed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC-ND 4.0) Licence (https://creativecommons.org/licenses/by-nc-nd/4.0/). Any third-party copyright material present remains the property of its respective owner(s) and is licensed under its existing terms.

Take down policy
If you consider content within Bath's Research Portal to be in breach of UK law, please contact: openaccess@bath.ac.uk with the details. Your claim will be investigated and, where appropriate, the item will be removed from public view as soon as possible.

Download date: 05. May. 2023

Investigation Into Microbiological And Biochemical Factors That Influence The Maturation Flavour Of Strong Cider

submitted by

Claire Helen Swaffield

for the degree of Ph.D.

of the

University of Bath

1996

Attention is drawn to the fact that copyright of this thesis rests with its author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the prior written consent of the author.

This thesis may not be consulted, photocopied or lent to other libraries without the permission of the author for three years from the date of acceptance of the thesis.

Signature of Author.....

Claire Helen Swaffield

UMI Number: U601907

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U601907
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF SURREY
LIBRARY
34 23 AUG 1996
PHD
510518

SUMMARY

Long term storage of fermented beverages, such as cider, significantly alters the final flavour characteristics of the product. Indeed, in most cases, some degree of maturation is required to improve product quality. The extent of change is, however, unpredictable, as the nature and identity of those factors responsible for the biochemical transformations are unknown. As a consequence, the cider maturation process occurs without any control, resulting in variable maturation times and inconsistent product flavour. Identification of those factors responsible for the maturation of fermented cider would bring significant benefits through standardisation and possible acceleration of this process.

Qualitative and quantitative examination of changes in microflora and flavour compounds during storage was undertaken in this research. Several potential maturation marker compounds were determined. Factors influencing cider maturation were thus found to be a result of complex interactions between chemical, physical and more importantly, microbiological parameters.

Acknowledgements

I am indebted to my industrial supervisor, Basil Jarvis, firstly for allowing the opportunity to carry out this research and for his unfailing encouragement and enthusiasm throughout the duration of the work, and for his constructive comments, when faced with seemingly endless pages of text.

I wish to thank Ashley Scott, my academic supervisor, for his guidance and support during the last four years. I would also like to take this opportunity to thank Ann O'Reilly and Fernando Acosta at Bath University for their assistance in the laboratory.

My thanks extended to BBSRC (formerly SERC) and H. P. Bulmer Ltd. for their financial support.

I am grateful to all the staff at H.P. Bulmer who have helped me and offered their know-how and advice. Special thanks to Loraine Boddington for her patience and persistence, in particular in matters concerning wordprocessors and 'ancient' references. Terry Campbell also deserves a special mention for his perseverance with the GC.

I also thank my family for not looking too puzzled when I attempted to explain what I've been doing with cider (apart from the drinking of it) and for their continuing belief in my abilities.

I am much obliged to Mike for making my computer well again at such short notice and Rob for developing multiple copies of the micrographs included in this thesis.

There have been a number of friends and colleagues who deserve words of gratitude for their constant encouragement, friendship and ability to make me laugh no matter what. In particular, I thank Philippa and Elaine at Bath and Sue, Grace and Amalka at Bulmers. The last words are for Mark, for not letting me throw the computer out of the window and for always being here for me, in whatever capacity was required.

CONTENTS

CHAPTER ONE

INTRODUCTION

Alcoholic beverages are produced virtually world-wide in what has become a very diverse industry. The basic methods of potable alcohol production however, follow the same general principles. Alcoholic beverage production is based on the formation of ethanol, together with a wide range of quantitatively minor, but organoleptically important compounds,primarily by *Saccharomyces* species, usually *Sacch. cerevisiae* or *Sacch. uvarum.*

The first recorded manufacture of cider is in the Twelfth Century, at a site in Normandy. During the following four centuries, extensive planting of apple orchards was undertaken, with the golden era of cider making in the Seventeenth Century. After the Napoleonic Wars, however, corn growing and cattle raising become a more profitable occupation and the cider industry went into a gradual decline. It was not until 1875 that a revival was started. In 1912, the Agricultural Research Institute was set up at the University of Bristol at which information was collected on pomology and fermentation. Problems of fruit culture, control of diseases and pests of fruit trees, and the development of cider making were also examined. This research helped transfer cider making from an essentially farm based enterprise to industrial scale and ensured its growing popularity with the modern consumer (Beech, 1972b).

1.1 MODERN METHODS IN CIDER MAKING

1.1.1 Fruit and Juice

Cider (or cyder) is the fermented juice of the apple, *Malus pumilla.* England is the major producer of cider, although several countries also produce this beverage. In the south and west of England, north west France and northern Spain, the true cider apple, together with outgraded culinary apples are used in cider making. In Switzerland, Germany, Australia and North America (hard cider), outgraded dessert and culinary apples are used (Beech, 1993a). Most cider apple orchards in Britain are planted in collaboration with the large

cider manufacturers, with Bulmers Norman, Dabinett, Yarlington Mill and Sweet Alford being some of the most common varieties grown.

Modern cider production is no longer a seasonal industry owing to the utilisation of both fresh and concentrated fruit juices. Concentrate used in the UK is often a mixture of juices from home grown and French cider apples, outgraded English dessert apples and culinary apples. Apple juice concentrate is more stable than fresh juice as it has a high osmotic potential and a low water activity. It is, however, still subject to some chemical and biological changes, unless appropriate storage conditions are employed. The fruit is milled to a pulp and the juice extracted by either hydraulic pressure or centrifugal force. Juice composition may then be standardised by the addition of sugar and malic acid, while spoilage micro-organisms may be controlled by addition of sulphur dioxide.

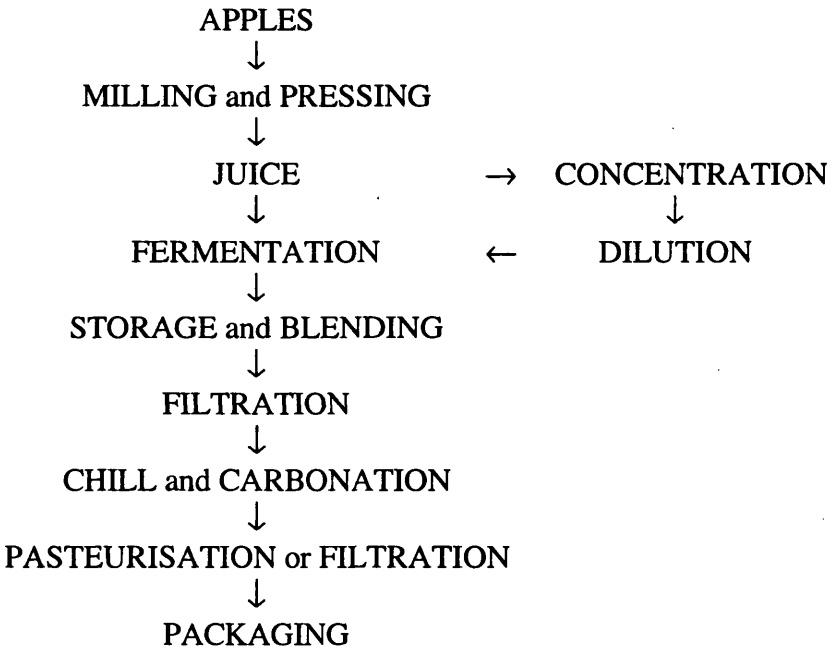

Figure 1.1 Flow diagram of the process of cider making

1.1.2 Sulphur Dioxide

Flavour and aroma of apple juice can be modified, usually unfavourably, by an increasing population of micro-organisms. For this reason, the microflora is often controlled as soon as the juice is expressed from the pulp by addition of sulphur dioxide. Micro-organisms vary greatly in their sensitivity to sulphur dioxide. Aerobic organisms are more sensitive to

sulphur dioxide than fermentative organisms. The precise nature of the inhibitory action of sulphur dioxide on micro-organisms is however, still not completely understood (Beech, 1993b). An appropriate dose of sulphur dioxide is added to apple juice to counter any binding agents, whilst leaving sufficient free sulphur dioxide to destroy aerobic organisms. The process takes at least six hours. Anaerobic micro-organisms, such as *Saccharomyces* species, *Saccharomycodes ludwigii* and *Shizosaccharomyces pombe* survive. Sulphite resistance in yeast is associated with three factors: i) small accumulation of sulphite due to ii) relatively low intracellular pH and iii) relatively small intracellular buffering capacities (Mahoney, 1993).

Some lactic acid bacteria initially decrease in numbers, and exist in a dormant state for several weeks before recommencing growth (Beech, 1993a). A few species are resistant to sulphur dioxide, although this resistance varies from species to species, and even from strain to strain. The effectiveness of sulphur dioxide is also dependent on the pH of the medium, presence of sulphite binding compounds (as previously mentioned) and the oxygen content of the apple juice. Correctly used, sulphur dioxide has a marked effect on the microflora, reducing the total number of viable micro-organisms to 1.25% of the original value (Beech and Carr, 1977), destroying more bacteria than yeasts. Sulphur dioxide has several unique properties: it is an antioxidant, an inhibitor of oxidising enzymes, combines with products of previous oxidation, prevents darkening and some hazes, as well as being inhibitory to a wide range of micro-organisms.

When sulphur dioxide is added to apple or grape juice, it equilibrates into a mixture of molecular sulphur dioxide, bisulphite and sulphite depending on pH. This reaction is dependent on both temperature and pH value of the apple juice and cider. Free sulphur dioxide is the proportion of sulphur dioxide present as molecular sulphur dioxide and the bisulphite ion. Molecular sulphur dioxide is the only form able to enter microbial cells and is therefore antimicrobial. Total sulphur dioxide, based on all forms of sulphur dioxide is the type specified in legislation and the legal limit for cider and perry is 200 ppm (HMSO, 1995).

1.1.3 Fermentation

The next stage is to add a pure culture of the requisite yeast strain and then allow the juice to ferment. In commercial operations, very little control is exercised over this process phase.

There are many factors involved in the process of cider fermentation. These control the rate and extent of fermentation and excretion of metabolic products by the yeast, which contribute to the characteristic flavour of the cider. Rate of fermentation is also influenced by any juice treatment. Composition of medium, oxygen content, yeast strain, yeast condition, pitching rate, fermentation temperature, carbon dioxide counter pressure, size and shape of the fermentation vessel, are all factors controlling fermentation (Torrent *et al*, 1989).

Fructose, glucose and sucrose are fermented by yeasts via the Embden-Meyerhof-Parnas pathway to ethanol and carbon dioxide. Yeast of the *Saccharomyces* species also produce organic acids and aroma compounds as by-products of the fermentation.

The fermentation proceeds in three stages: Lag, exponential and stationary. The lag phase length is directly related to the amount of sulphur dioxide added. It is at this stage that half the free sulphur dioxide is bound to juice constituents. During the lag phase there is also a strong uptake of soluble nitrogen, which continues throughout the intermediate phase. There is a corresponding increase of nitrogen in suspension at that time, which reaches a maximum as the soluble nitrogen content of the solution reaches a minimum. This continues until another nutrient becomes the limiting factor. As the specific gravity drops to between 1.005 and 1.010, there is an increase in soluble nitrogen, due to cessation of yeast growth and excretion of nitrogenous compounds, which consist of aromatic amino acids, peptides and nucleotides. Autolysis will also affect the soluble nitrogen content of the cider. A decrease in soluble phosphate compounds reflects the decrease in nitrogen, but phosphate compounds are more readily excreted by the cells into the medium, so there is an increase in phosphates earlier in the fermentation. The growth rate of yeast is not determined solely by the soluble nitrogen content of the base, but by a particular B group vitamin, thiamine, which is assimilated during the early stages of the fermentation and is sufficient for the whole course of yeast growth (Beech and Carr, 1977).

The flavour produced by a particular yeast culture is dependent on the pattern of its production of higher alcohols, esters and ketones (Beech, 1993b). Cider flavour, however, is assessed more by the absence of taints, a moderate level of aroma compounds, the degree of sweetness and acidity the consumers appreciate, and alcohol content (Beech, 1993b).

Prior to the use of sulphur dioxide to control the natural microflora of apple juice, the fermentation was allowed to proceed with the degradation of sugars by *Kloeckera apiculata*. These rapidly growing yeasts die out when the alcohol content increases to 2-5% v/v. The fermentation is then taken over by *Saccharomyces* species, which are dominant by the end of the fermentation (Salih *et al*, 1988). There is disagreement as to whether the apiculate yeast have a significant effect on aroma, or whether the aroma is due entirely to *Saccharomyces* species (Farris *et al*, 1991).

Pure culture inoculation fermentations have a more rapid onset of fermentation, higher alcohol yields and a clearer product, compared with natural fermentations. The product also has an improved aroma and a distinctive vinous character. The use of a pure culture offers many advantages, including ease of control and homogeneity of fermentation. It has been found that the volatile flavour components synthesised by natural microflora are found in higher concentrations in cider fermented by pure cultures, while the opposite is true for substances detected in low concentration (Moreno *et al*, 1991). It is now of great importance that the best yeast strain is chosen for the requirements of the cider maker as the strain has a marked influence on fermentation rate, with non-flocculent yeasts fermenting sugars more rapidly than flocculent yeasts. It is generally considered that, although the rate of fermentation is governed by the amount of yeast in suspension, the yeast in the lees do continue to take an active part in the fermentation (Beech and Carr, 1977).

The chosen culture must be pure and stable, against both formation of respiratory deficient mutants and changes in flocculation patterns. The yeast must be resistant to sulphur dioxide. The chosen strain should not produce unwanted metabolic products, nor be reliant on an external source of vitamins and should have an efficient production of ethanol. It is desirable that the yeast should have a suitable flocculating pattern, should produce fusel alcohols and organic acids in appropriate quantities and should be capable of degrading pectic acid to galacturonic acid (Beech, 1972b).

1.1.4 Storage/Maturation

Most ciders benefit from a holding period after fermentation, allowing the yeast to settle out, forming the lees, and the elimination of diacetyl. The cider can then be removed from the yeast deposit, that is racked off the lees (Beech, 1993b). Ciders in which malo-lactic fermentation is required are preferably held in wooden vats (Beech, 1993b). When ambient temperature reaches 15°C any lactic acid bacteria present may deacidify the cider by a secondary fermentation.

Cider is then stored in the absence of air until required. A series of biochemical and microbiological processes are brought about at this stage, changing the composition of cider (Yurchenko *et al.*, 1974, Williams, 1989). The aim of maturation is to reduce harsh flavour notes and produce a more mellow product (Pollard *et al.*, 1966). Conditions, however, under which these processes are best achieved remains an area for empirical judgement, as very little is known about the changes taking place during this storage period.

Depending on storage conditions, several chemical reactions influence the volatile composition of cider. Changes in equilibrium between esters, acids, alcohols and carbonyls occur even during a short period of storage (Williams and Tucknott, 1978). Esters may be hydrolysed until their concentration approaches equilibrium with their component acid and alcohol, except when the acid or alcohol is in excess, due to microbial action (Beech and Carr, 1977). There is generally a decrease in lower aliphatic acid esters, for example, ethyl butanoate, ethyl octanoate, ethyl decanoate, propyl acetate, 3-methylbutyl acetate and hexyl acetate (Williams, 1989) and a corresponding increase in mixed esters, such as 3-methylbutyl-3-methyl butanoate, ethyl lactate and diethyl succinate. Ethyl esters hydrolyse slowly due to the high ethanol content in cider. Organic acids remain static or increase through biosynthesis. Generally, there is an increase in concentration of higher boiling point compounds.

Finally, cider is stabilised by fining and filtering, blended to obtain a standard product, and pasteurised prior to packaging. Factory ciders are sweetened and carbonated prior to bottling, canning or kegging.

1.2 FLAVOUR

Cider flavour, in common with other fruit based alcoholic beverages, is affected by fruit type, fruit quality, juice preparation, the nature of the fermentation, the type of micro-organisms present and post fermentation treatments. Over the past 20 years, commercial cider flavour in the UK has changed from one of a bittersweet apple taste to a clean, fresh taste (Beech, 1993b).

Flavour is the result of complex interactions of the cider's chemical constituents with the sense of taste and smell of the consumer (Williams, 1974). Such constituents vary from nanogram to grams per litre quantities (Moir, 1992). Non-volatile compounds bestow the taste sensations upon the consumer (Rapp and Mandery, 1986). The aroma is a balance of several hundred compounds, largely made up of higher alcohols with smaller amounts of esters and other components (Pollard *et al*, 1966). These aroma constituents vary with pre- and post-fermentation treatments. The main aroma arises during the fermentation conferring a vinous character to the product. Some stabilisation treatments, such as filtration and fining, result in the apparent loss of aroma due to binding of small molecules by macromolecules (Voilley *et al*, 1990).

The number of flavour sensations that can be appreciated by the palate is limited: sweetness, sourness, saltiness, bitterness, astringency, metallic taste and pungency. Flavour is composed of volatile compounds, for example, alcohols, esters, aldehydes, ketones and hydrocarbons as well as of non-volatile compounds. Organic acids, sugars, phenolic compounds and mineral substances are examples of such compounds (Schreier, 1979). The major part of the flavour character is perceived through the nose and full understanding of flavour quality will come from the integration of developments in analytical chemistry with sensory characterisation (Williams, 1982).

Flavour components originate from various sources and may be influenced by a variety of factors. Certain components are within the fruit itself and persist throughout processing and contribute to the final product flavour, but an extensive number of flavour components are synthesised during fermentation. Finally, post fermentation treatments, such as

clarification, blending, distillation, fortification and ageing all have an effect (Williams, 1982).

1.3 CIDER RESEARCH

1.3.1 Past and Present

The cider industry is undergoing a revival at present, hence research into cider production techniques is becoming an important issue for the cider manufacturers, as they are competing with the technically more advanced brewing industry. The increasing rate of cider consumption clearly indicates a growing industry producing good, clean cider in quantity, but there has been a loss of many of the good characteristics associated with the best of old ciders. Modern ciders have sometimes been said to lack flavour and character (Williams, 1974) and hence manufacturers are looking for a means of improving product quality and increasing their control over cider flavour. Ultimately, as knowledge of cider flavour increases, it should be possible to vary cider flavour such that a cider can easily be adapted to suit smaller segments of the population and fluctuations in flavour demands.

An intimate knowledge of constituents, both volatile and non-volatile, their effect on cider flavour, their biochemistry and effect of manufacturing variables is required to permit definition of parameters for flavour control and explain 'taints'. Gas chromatography and other analytical techniques have enabled the accumulation of vast amounts of information, such that a large number of compounds present in cider are known, together with biosynthesis information and factors affecting their production. Much of the information regarding compound biosynthesis is from the brewing, spirits and wine industries. This is only of assistance when the flavour significance of individual compounds can be evaluated and analytical information correlated with flavour.

A substantial amount of research was undertaken at Long Ashton Research Station between 1903 and 1986. Investigations on a range of issues were undertaken, such as composition of apples, juices and ciders, their microflora, factors controlling rate of fermentation, usage of sulphur dioxide, flavour quality and consumer acceptance (Beech, 1993a). In the 1970's, a consortium of the major cider producers agreed to fund aspects of cider and pomological research (especially phenolics using HPLC analysis to determine

concentrations of individual compounds). Since the closure of this Food and Beverage Division in 1986, research has been primarily funded by industry. Research has concentrated on integrated pest management methods, fate of agrochemical residues during processing and fermentation, factors affecting formation of sulphur dioxide-binding compounds and improvements in chemical and microbiological methods of analysis.

Currently, in the UK, product development, handling, processing and packaging equipment and techniques, (for example, temperature controlled stainless steel tanks), yeast characteristics, fermentation biochemistry, stability and standardisation of final product are all being investigated (Beech, 1993a).

In France, cider research is centred on quantitative evolution of volatile aroma compounds by different yeast strains (Leguerinel *et al.*, 1988; Leguerinel *et al.*, 1989), yeast flora in French cider factories (Salih *et al.*, 1988), microflora development in natural fermentations and their interactions and influence in cider flavour (Michel *et al.*, 1988; Michel *et al.*, 1990). In Spain, yeast populations during natural fermentation, rate of fermentation of apple juice undergoing different processing techniques (Cabranes *et al.*, 1990) and malo-lactic fermentation (*Salih et al.*, 1987; Salih *et al.*, 1990) are being studied.

1.3.2 This Research Programme

During long term storage of cider, final product quality is generally improved and significant alteration of the flavour profile is observed. This maturation process is uncontrolled, resulting in unpredictable storage periods and variable final product. In order to standardise this storage phase, the factors responsible for changing the component profile have to be determined. These may include microbiological, chemical or physical factors, or a combination thereof. Successful identification of significant marker compounds and the factors involved in their formation or depletion will provide a reference from which a controlled and standardised post fermentation process can be developed.

As a consequence, a research programme was conceived in two phases: Firstly, observations of commercial scale cider storage, as traditional wooden storage vats are disappearing, along with associated flavour characteristics; Secondly, laboratory scale investigations into the effects various storage parameters have on cider flavour.

Commercial Cider Storage

Microbiological, organic acid and sugar profiles of commercial cider stored in a traditional wood vat were observed over a two and a half month period, along with an appraisal of selected volatile flavour components during this time. Temperature, pH and dissolved oxygen content of the cider were recorded at this time.

Microbial ecology of cider storage vats was assessed. Both wood and epoxy resin lined concrete vessels were scrutinised for persistence of micro-organisms after cleaning. Microbial colonisation of wood vats was observed and subsequently studied. The ability of these indigenous micro-organisms to influence cider flavour was examined.

In addition, micro-organisms were isolated from a variety of storage ciders which were at different stages of maturation. Vessels of various construction materials were examined over two different seasons. These micro-organisms were utilised for laboratory scale studies.

Laboratory Cider Storage

Effects of temperature, filtration/pulp particle size, dissolved oxygen, dissolved carbon dioxide and agitation on volatile flavour components were examined.

Micro-organisms isolated from commercial ciders during different stages of storage were purified and maintained. The bacterial isolates were characterised to genus and reintroduced into cider to assess their ability to influence flavour as pure cultures.

1.4 SUMMARY

- Research into cider production techniques is becoming an important issue for the cider manufacturers, as they compete with the more technically advanced brewing industry.

- Manufacturers are, therefore, looking for a means of improving product quality and increasing their control over cider flavour.

- A loss of many good flavour characters associated with the best of old ciders has been the penalty of large scale production of standardised cider.

- An intimate knowledge of constituents, both volatile and non-volatile, their effect on cider flavour, their biochemistry and effect of manufacturing variables is required to be in a position to give parameters for flavour control and explain 'taints'.

- Pomology and fermentation optimisation, and their effects on flavour, has been and is currently the main thrust of research in the cider industry.

- The purpose of the work described in this thesis was to overview the influence of biochemical and microbiological factors on cider flavour during storage.

CHAPTER TWO

LITERATURE SURVEY

The vast majority of alcoholic beverages undergo some form of maturation or ageing. Storage of cider brings about a series of chemical, biochemical and microbiological processes, which change the composition of the cider (Yurchenko *et al.*, 1974; Williams, 1989). The aim of maturation is to reduce the harsh flavour notes and to produce a more mellow product (Pollard *et al.*, 1966). The conditions under which these processes in cider are best achieved remains an area for empirical judgement as nothing is yet known about the changes taking place during the period of storage, although it is well observed that many do occur.

Generally, racked cider is stored in large vats until it is required for packaging, with little regard for flavour improvement, hence it is considered by the cider makers as a period for preventing deleterious changes. The headspace of the vat is filled with carbon dioxide, nitrogen or a mixture, as film yeast and acetic acid bacteria will grow during storage, if the cider is exposed to air. Stored cider may be regarded as the raw material of the cider maker and blended accordingly, to produce a standard, pleasing product.

Depending on the storage conditions, several chemical reactions influence the volatile constituent composition of the cider. Changes in equilibrium between esters, acids, alcohols and carbonyls occur even during a short period of storage (Williams and Tucknott, 1978). Major equilibration occurs during storage, with an increase in concentration of higher boiling point compounds due to the evaporation of the lower boiling point components, acetal formation and esterification. Esters are generally hydrolysed during storage until their concentration approaches equilibrium with the component acid and alcohol. The exception to this is when the acid or alcohol component is in excess due to microbial action.

This research project is concerned with flavour modifications during cider storage and factors influencing these changes. A considerable amount of work has been published on

flavour compounds in cider, in particular the volatile components of flavour. A significant understanding of flavour compound synthesis during cider fermentation has also been achieved. Similar studies have been pursued in wine research and many similarities between the two fruit-based alcoholic beverages have been determined, as well as differences. Maturation of wine and the associated causes and effects has, however, been studied to a far greater depth of understanding than cider maturation. Thus, research into cider maturation will draw upon information from investigations concerning wine maturation, while recognising that both the products and their production methods are distinct.

2.1 CIDER FLAVOUR

2.1.1 Non-Volatile Compounds

Non-volatile compounds, as well as also influencing flavour, bestow the taste sensations upon the consumer giving rise to the mouthfeel of the product (Rapp and Mandery, 1986). Cider contains sugars, acids and phenolics, as well as amino acids, nucleotides, vitamins and metallic ions, all of which influence the consumer's perception of a product, to greater or lesser extent.

Sugars

Apple juices contain between 90-120 g/l in total of fructose, glucose, sucrose and traces of xylose (Salih *et al.*, 1988). Fructose occurs in concentrations between 6-10% while glucose and sucrose are less abundant, usually being present in concentrations of 1-2% (Carr, 1959). Fructose, glucose and sucrose are fermented by yeasts of the *Saccharomyces* species, via the Embden-Meyerhof Pathway, to ethanol and carbon dioxide. It is unlikely, however, that sucrose is present in any significant quantity at the beginning of fermentation, as it is rapidly inverted at this time (Carr, 1959). Non-fermentable sugars, in particular sorbitol and other hexitols, are also present.

Dry cider contains traces of glucose and fructose but more importantly, there are a number of sugars and sugar-like compounds, such as xylose, sorbitol, glycerol, mannitol, inositol, ribose, galactose, sorbose and rhamnose that are not fermented by yeasts. Many of these compounds can however, act as energy sources for bacteria during storage.

Glucose is converted to lactic acid, ethanol and carbon dioxide by lactic acid bacteria, whilst fructose is reduced to mannitol, lactic acid and acetic acid (Carr, 1959; Beech, 1972b; Tracey and van Rooyen, 1988). Fructose is utilised for two purposes, first as an energy source (Kunkee, 1967) and secondly as a hydrogen acceptor (Beech and Carr, 1977). It has been reported that pentose sugars, particularly fructose are used by these bacteria with greater efficiency than glucose (Carr, 1959; Kunkee, 1967). Tracey and van Rooyen (1988) reported that additional lactate and acetate is formed from glucose and fructose respectively, in the presence of malic acid. Glucose and L-malic acid utilisation occurs simultaneously, but the latter is degraded at a much higher rate than glucose as a result of an increase in sugar catabolism induced by the increase in pH of the medium achieved by malic acid degradation and the increase in synthesis and activity of D-lactate dehydrogenase (Firme *et al.*, 1994). In the presence of citrate, however, there is an increased formation of acetate from glucose, due to the formation of pyruvate after citrate has been split (Firme *et al.*, 1994).

Organic Acids

Organic acids constitute one of the main taste groups in cider, contributing sourness to a product. All organic acids found in alcoholic beverages have this quality to some degree, but some have their own characteristic flavour, taste or aroma; for example, succinic acid has a salty, bitter taste and citric acid has a fresh acid flavour.

A variety of organic acids are found in apple juice, the most significant being L(-)malic acid. Other acids found include pyruvic, oxaloacetic, α-ketoglutaric, citramalic (associated with apple peel), fumaric (associated with green apples), D-gluconic, and 2-methyl-2:3-dihydroxybutyric acid. Citric acid, found in trace amounts in apples, can influence the metabolic activities of lactic acid bacteria. Malic, succinic and pyruvic acids are formed by cider yeasts over the pH range 3.4-4.0 (Beech, 1993b). *Saccharomyces* strains vary in their ability to produce or metabolise organic acids (Beech, 1993b). Other acids formed during fermentation include lactic, hydroxyglutaric, 2-ketoglutaric, fumaric, gluconic, dihydroxybutyric and α-hydroxybutyric acids (Beech, 1993b).

The fate of many organic acids is often dependent upon bacterial action during the storage of the cider (Williams, 1974). Lactic acid bacteria are able to utilise carbon sources which

are not metabolised by yeasts. Thus, in the absence of sugars, organic acids are utilised by bacteria. The metabolism of malic acid by these bacteria, that is, malo-lactic fermentation, readily occurs in cider (Whiting, 1976). Indeed, malic acid stimulates malo-lactic fermentation (Velãquez *et al.*, 1991) while lactic, succinic and tartaric acids have an unfavourable effect on malo-lactic fermentation, the last reducing the amount of biomass formed (Lafon-Lafourcade and Ribereau-Gayon, 1984; Velãquez *et al.*, 1991). Lactic acid bacteria not only metabolise malic acid, but also citric, pyruvic, quinic, shikimic, chlorogenic and caffeic acids (Beech, 1972b; Williams, 1974). Acids are attacked in a specific order: Malic, then citric, quinic, shikimic and finally esters of quinic and hydroxy-cinnamic acids (Beech, 1972b).

Malic acid in apples decreases during storage prior to processing, and may either be broken down or formed during yeast fermentation (Beech, 1972b; Leguerinel *et al.*, 1989). Depending on yeast strain, pH and nitrogen content of the apple juice the concentration of this acid increases, sometimes by more than 50% of the original value (Beech, 1993b). Conversely, malate may be converted to ethanol and carbon dioxide. Low pH and high nitrogen content favour this breakdown (Whiting, 1976). *Saccharomyces* species possess an enzyme which oxidises malate to pyruvate and carbon dioxide (Schreier, 1979). Malic acid is also transformed to hydroxy acids, such as 2-methyl-2:3-dihydroxybutyric acid, 2:3-dihydroxy-*iso*-valeric acid (a valine precursor), 2-ethyl-2:3-dihydroxybutyric acid and 3-ethyl-2:3-dihydroxybutyric acid. It can also form lactic acid, by the action of yeast with glycerol (Farris et al., 1991).

Succinate is formed by yeast during alcoholic fermentation of glutamic acid with the reduction of oxalacetic acid. It may also be produced from malic acid in association with fumaric and lactic acid (Schreier, 1979). Final concentrations in cider vary from trace amounts to 1.6 g/l, while in wine the concentration range is from 0.57 to 1.82 g/l (Whiting, 1976).

Traces of the keto (oxo) acids pyruvic, α-ketoglutaric and oxaloacetic are formed during fermentation (Beech, 1993b). Pyruvate is found in cider in the range of 80-640 mg/l, while in wines a range of 11-460 mg/l is observed. 2-oxoglutaric acid has been found in the range 2-346 mg/l in wines and trace to 50 mg/l in ciders (Whiting, 1976). They are

intermediary products of the formation of higher alcohols from amino acids in yeasts (Schreier, 1979).

Formation of 2-hydroxycarboxylic acids is achieved during yeast fermentation from their corresponding amino acids. D-lactic acid is formed by all fermentation yeast strains under anaerobic conditions, especially if aspartate is added to juices of high pH or low in thiamine (Whiting, 1976). The absence of thiamine favours lactic acid production, as NADH is oxidised by reduction of pyruvate to lactate instead of acetaldehyde to ethanol (Whiting, 1976). Reduction of pyruvate results in the formation of either L- or D- lactate (Whiting, 1976). L-lactic acid is more commonly formed in cider by the action of bacteria (Schreier, 1979). Although it is very unusual for the yeast flora to degrade lactic acid, its degradation is identified with a progressive and simultaneous increase in malic, α-ketoglutaric and fumaric acids. Its appearance is correlated with acetic acid production, known as piqûre lactique (Beech, 1993b).

Medium chain length fatty acids, such as hexanoic, octanoic and decanoic acids are toxic fermentation by-products, which can completely inhibit yeast growth in the presence of ethanol (Munoz and Ingledew, 1989). Fatty acid biosynthesis commences with the formation of acetyl coenzyme A by oxidative decarboxylation of pyruvic acid. All intermediates in this synthesis are bound to a multienzyme complex until the fatty acids are formed. Propionyl coenzyme A replaces acetyl coenzyme A in the biosynthesis of odd numbered fatty acids (Suomalainen and Lehtonen, 1979).

Malic acid is fermented by both hetero- and homo-fermentative types to lactic and succinic acids. Malic acid is decarboxylated to pyruvic acid and carbon dioxide by enzymes in the presence of NAD (nicotinamide adenine dinucleotide) and manganese ions. Pyruvic acid is then reduced to lactic acid by lactate dehydrogenase and NADH (Benito de Cardenas et al., 1991a; Benito de Cardenas et al., 1991b; Nuraida and Owens, 1992; Tsau *et al.*, 1992). Here, pyruvic acid acts as a hydrogen acceptor (Kunkee, 1967). Some bacteria convert malic acid to lactic acid and carbon dioxide and others, at higher pH values, convert malic acid to succinic and acetic acids and carbon dioxide. Yet others convert malic acid to acetoin and diacetyl (Williams, 1974). The exact type of acid produced when these

organisms convert malic acid to lactic acid varies with pH. At lower pH levels, succinate and lactate are produced, but at pH 4.8, only succinate is produced (Beech, 1972a).

Pyruvate is a key catabolic intermediate for many lactic acid bacteria and is converted into a variety of end products, such as lactic acid, acetic acid, acetaldehyde, ethanol, diacetyl and acetoin. Pyruvate is a precursor to three related compounds: diacetyl (2,3-butandione), acetoin (3-hydroxy-2-butanone) and butylene glycol (2,3-butandiol). Heterofermentative lactic acid bacteria metabolise pyruvate only when all the glucose has been exhausted (Benito de Cardenas *et al.*, 1991b; Nuraida and Owens, 1992). It is suggested that this is due to inhibition of acetolactate synthase activity by intermediates of glucose metabolism. This inhibition is somewhat reduced at low pH, which accounts for greater production of acetoin and diacetyl at low pH values (Benito de Cardenas *et al.*, 1991a; Nuraida and Owens, 1992). Another product of pyruvic acid metabolism in some bacteria is formic acid (Kunkee, 1967).

Citric acid is mainly fermented by heterofermentative rods and cocci, although *Pedicoccus cerevisiae*, *Lactobacillus buchneri* and *Lact. delbruckii* are unable to attack this acid. It was determined that lactic acid bacteria could have an induced or constitutive enzyme that was strain specific rather than being related to their homo- or heterofermentative character (Beech, 1972b; Kunkee, 1967). Bacteria with a constitutive malic enzyme could metabolise citrate by the action of an induced citratase. Those isolates, such as *Lact. plantarum*, which possess a constitutive malic enzyme can metabolise citrate by citratase (Benito de Cardenas et al., 1991b) via the pathways below (Kunkee, 1967).

2 Citrate

↓

2 Oxalacetate + 2 Acetate

↓

2 Pyruvate + 2 CO_2

↙ ↘

Acetoin + CO_2 **Lactate + Acetate + Acetoin + Diacetyl + CO_2**

(Homofermenters) **(Heterofermenters)**

Leuconostoc spp. produce lactate and acetoin from citrate. Acetic acid and diacetyl are also formed. Thus, with high concentrations of citrate, acetification can take place anaerobically, tainting the product (Beech and Carr, 1977). It is important that malo-lactic fermentation is avoided where the citrate content exceeds 0.05% (Beech, 1972b).

Phenolics

Many English ciders are characterised by their bitterness and astringency, which are associated with phenolic compounds. Astringency produces a physical sensation caused by the interaction of tannins with proteins or glycoproteins of the mouth, whereas the causes of bitterness are still not well understood (Lea and Timberlake, 1974).

Procyanidins (flavan-3-ols) are the main contributors of bitterness and astringency to the cider. Together with leucoanthocyanidins (flavan-3,4-diols), they are known as leucoanthocyanins. Leucoanthocyanins are found in the fruit, although flavan-3,4-diols are not commonly detected in apple juice as they are very unstable (Lea and Timberlake, 1974). Procyanidins are polymers based on catechin and epicatechin units. Although all procyanidins isolated from cider are both bitter and astringent, their sensory impact and character vary. In principle, polymers of four or more catechin units are actively bitter or astringent at concentrations occurring naturally in cider. The tetramers are the most bitter and the more polymeric material the most astringent (Lea and Timberlake, 1974; Williams, 1982).

Phenolics are adversely affected by, and very susceptible to, both enzymic and non-enzymic oxidation. Polyphenol oxidase and phenolase are the enzymes that cause polymerisation of procyanidins, thus `tanning' cell debris by a polyphenol-quinone-protein reaction. The resultant juice is less bitter and more astringent (Jones *et al.*, 1986). In general, oxidation is avoided in order to attain maximum mouthfeel, as well as to prevent undesirable colour changes, especially browning. Phenolic acids, shikimic and quinic, as well as chlorogenic and p-coumaryl acids, are commonly found in bittersweet apple juice (Williams, 1974). When the cells of the fruit are ruptured by milling and pressing, the phenolics combine with proteins present in the juice (Beech and Carr, 1977).

The final concentrations of these components are influenced by yeast and bacterial activities. Yeast are able to decarboxylate ρ-coumaric acid, ferulic acid and vanillin (Suomalainen and Lehtonen, 1979) and form 4-ethylphenol and 4-ethylguaiacol from coumarin and caffeic acid, respectively (Williams and Tucknott, 1971). Lactic acid bacteria are capable of attacking quinic, shikimic, chlorogenic and other acids that occur in ciders (Beech, 1972b). These bacteria are able to oxidise or reduce quinate or shikimate under anaerobic conditions (Beech, 1972b). Quinic acid is reduced to dihydroshikimic acid immediately after malo-lactic fermentation (Beech, 1972b). Metabolism of quinate by heterofermentative rods passes through the stages of 3-dehydroquinate, 3-dehydroshikimate, shikimate and dihydroshikimate (Beech and Carr, 1977). In contrast, the homofermentative rod *Lact. plantarum* continues the metabolism of quinate to 3,4-dihydroxycyclohexane-1-carboxylate and also produces catechol, which tends to give an off flavour (Beech and Carr, 1977). In the presence of fructose, quinate and shikimate are converted to dihydroshikimate, acetate, water and carbon dioxide. In this case, the fructose acts as a proton acceptor, trebling the energy made available from fructose (Benito de Cardenas et al., 1991a). Bacterial growth is thus achieved in the presence of shikimate or quinate, in so doing, suppressing the formation of lactate and mannitol (Beech, 1972b; Beech and Carr, 1977). While quinate and shikimate have the advantage of increasing the amount of energy available to the organism, the disadvantage of a six-fold increase in acetate on flavour has to be considered (Beech, 1972b; Beech and Carr, 1977).

Chlorogenic acid may be transformed by *Lact. collinoides* to quinic and caffeic acids. The former compound eventually forms ethyl catechol, while the latter is metabolised to ethyl catechol, via dihydrocaffeic acid (Beech and Carr, 1977). ρ-Coumarylquinic acid is converted to ρ-coumaric acid, which is later converted to ρ-ethylphenol, and quinic acid (Tsau *et al.*, 1992). Production of catechol and acetic acids from shikimic and quinic acids and production of ethyl catechol from chlorogenic acid spoils cider flavour (Beech, 1993b).

Amino Acids and other Nitrogen Compounds

Nitrogenous matter in cider occurs in concentrations of 5-15 μg/ml. The most easily detected nitrogen compounds are amino acids and as a consequence more is known about them than any other nitrogen source.

There are a number of amino acids in apple juice, principally asparagine, aspartic and glutamic. Indeed, asparagine may account for up to 50% of the total soluble nitrogen content of apple juice. Small to medium quantities of serine, α-alanine, γ-aminobutyric acid, methylhydroxyproline, valine, and isoleucine are also present. Others appear in trace amounts (Beech and Carr, 1977).

During fermentation, apple juice amino acids disappear as they are metabolised by yeast, only to reappear later as a result of yeast excretion and autolysis, in smaller quantities but in greater variety (Beech and Carr, 1977). Yeast autolysis is associated with an enrichment of the medium with free amino acids, total nitrogen, ammoniacal nitrogen, ribose, phosphate and Group B vitamins. It is also associated with the appearance of unpleasant odours (Chatonnet *et al.*, 1991). Autolysis therefore has the potential of enhancing bacterial growth. Amino acids typical of autolysis are the leucines, lysine, arginine, phenylalanine and tyrosine. There is also a constant interchange of amino acids brought about by yeasts and bacteria, as they are able to excrete some amino acids, such as α-alanine and phenylalanine (Beech, 1972b; Beech and Carr, 1977). Many lactic acid bacteria have an absolute requirement for valine, probably a growth controlling factor, which is present in low levels in cider. Arginine, isoleucine, glutamic acid and tryptophan are essential for their growth. Leucine is essential for between 45% and 75% of strains. Cysteine is required by the majority of strains, although tyrosine and phenylalanine are not essential (Fourcassie *et al.*, 1992). Growth is enhanced by a deficiency in proline, glycine or serine (Fourcassie *et al.*, 1992). Deficiencies in leucine, histidine or valine slow the rate of L-malic acid degradation, as do deficiencies in phenylalanine, proline, glycine or tyrosine (Fourcassie *et al.*, 1992).

Decarboxylation of amino acids by lactic acid bacteria leads to the formation of amines, which are undesirable (Radler, 1992). Arginine is probably converted to ornithine, which has been implicated indirectly in accelerated yeast death (King and Beelman, 1986). Histidine may be decarboxylated to histamine (Davis *et al.*, 1986). *Pediococcus* spp. are considered important producers of histamine, even when non-proliferating (Lafon-Lafourcade and Ribereau-Gayon, 1984; Davis *et al*, 1985; Radler, 1992). A strain of *Leuc. oenos* has been shown to decarboxylate tyrosine to tyramine (Radler, 1992). Williams

(1982) reported that although amines and amides are present (in wine), it is doubtful if they make any direct contribution to flavour.

Heterocyclic nitrogen compounds of interest in cider are the δ-piperidines which are believed to be associated with "mousy" off flavours (Williams, 1982). "Mousy" taint occurs periodically in ciders made from high pH juices, inadequately sulphited and infected with either *Brettanomyces* species or heterofermentative lactic acid bacteria. The flavour becomes apparent when the cider is exposed to air. This unpleasant taint in cider has been attributed to an alkyl substituted δ-piperidine, while in wine 2-acetyltetrahydropyridine and its imino tautomer have been proposed as the causative components (Beech, 1993b). These compounds are only formed by *Brettanomyces* yeast in the presence of lysine or by lactic acid bacteria in the presence of ethanol (Beech, 1993a).

Vitamins

Apple juice and cider contain a number of vitamins, of which those of the B group vitamins are the most important. Thiamine in particular, is influential as it determines yeast growth rate. It is assimilated during the early stages of the fermentation. The higher the concentration of this vitamin, the faster the fermentation rate (Beech and Carr, 1977). Some thiamine is released back into solution at the end of the fermentation. Nicotinic acid initially follows the same pattern of uptake as thiamine, but it is never completely depleted and more is released by yeasts into the medium as fermentation slows down. Yeasts provide vitamins for lactic acid bacteria (Leroi and Pidoux, 1993a).

In apple juices with low soluble nitrogen, there is a higher concentration of the vitamins riboflavin and pantothenic acid, than in high nitrogen level apple juices. These compounds gradually increase in concentration throughout the fermentation (Beech and Carr, 1977). Nicotinamide, pyridoxine, cobalamine, folic acid and biotin are also present in apple juice.

Lactobacilli require thiamine and riboflavin for growth, while cocci have a requirement for folic acid (Lafon-Lafourcade and Ribereau-Gayon, 1984). Kunkee (1967) reported that pantothenate was universally required by lactic acid bacteria, in relatively large concentrations, while nicotinic acid ranks second in importance (Carr, 1958; Beech, 1972a; Beech and Carr, 1977).

Thiamine may have a stimulatory effect on some lactic acid bacteria, while not being essential for their growth (Carr, 1958). Biotin has been reported to be an inhibitory agent to some *Leuconostoc* species (Carr, 1958).

Inorganics

Cider contains a selection of inorganics, which are either in aqueous solution as the metallic ions or complexed with cider constituents. Sodium, potassium, calcium, magnesium, iron, copper and phosphorus are the most common.

2.1.2 Volatile Compounds

Volatile components of any food or beverage are of prime importance to the understanding of its flavour. The use of gas chromatography, especially linked with mass spectroscopy, infra red spectroscopy and preparative gas chromatography has greatly influenced the understanding of cider flavour.

The flavour contribution of the alcohols, esters, acids, carbonyls and acetals is very complex. Many compounds are common to all fermentations, while others are perceived to impart a characteristic aroma to the product; for example, 2-phenyl ethanol, its esters and lower fatty acids are said to confer the basic cider aroma to the product, and aliphatic esters, in particular ethyl-2-methyl butyrate and hexyl acetate, give the cider its fruity, apple-like aroma. Certain ciders are associated with a heavy phenolic aroma, which is due to 4-ethylphenol and 4-ethylguaiacol. Aroma is largely caused by higher alcohols with smaller amounts of esters and other compounds, the amounts of all of which vary widely from one fermentation to the next (Pollard *et al.*, 1966).

Cider aroma is a balance of several hundred compounds, largely made up of higher alcohols with smaller amounts of esters and other components (Pollard *et al.*, 1966). These aroma constituents vary with pre- and post-fermentation treatments. The main aroma arises during the fermentation conferring a vinous character to the product. Some stabilisation treatments, such as filtration and fining, result in the apparent loss of aroma due to the binding of small molecules by macromolecules (Voilley *et al.*, 1990).

Alcohols

Higher alcohols, or fusel alcohols, are quantitatively the most significant components of all alcoholic beverages, though are only of secondary importance to the characteristic flavour of ciders. Desirable higher alcohol content varies according to the product: Beers are fermented in such a way as to minimise the synthesis of these compounds, with lager containing 50-80 ppm of higher alcohols and beer up to 200 ppm. Their production is encouraged in wines, as they are associated with good quality, imparting an amyl aroma to the product and can be found in concentrations up to 500 ppm. Cider has between 116 and 255 ppm of higher alcohols (Pollard *et al.*, 1965).

Concentrations of higher alcohols in cider vary according to yeast flora, juice treatment and fermentation conditions (Beech, 1993b). The presence of pulp particles in the juice enhances their production. This is considered to be due to oxygen entrapment by the particles (Pollard *et al.*, 1965; Pollard *et al.*, 1966; Klingshirn *et al.*, 1987). Indeed, any process that increases oxygenation of the juice will have a favourable effect on levels of higher alcohols (Beech, 1972a; Williams, 1974; Klingshirn *et al.*, 1987). Temperature of fermentation is also important (Williams, 1974) with higher temperatures increasing synthesis of butanols and pentanols, but reducing production of propanol (Leguerinel *et al.*, 1989). Conversely, any process that decreases the dissolved oxygen content of apple juice, such as sulphiting, pasteurisation, aroma stripping or enzymic treatments, decreases higher alcohol production. Thus, cider made from concentrate has less higher alcohols than ciders made with fresh juice (Beech, 1993b).

Higher alcohols are formed as the products of yeast metabolism of keto acids rather than amino acids (Williams and Tucknott, 1971) as the levels of the latter are very low in cider (Pollard *et al.*, 1965; Pollard *et al.*, 1966). Aromatic alcohols, such as tyrosol and 2-phenylethanol, arise through yeast metabolism of shikimic acid (Pollard *et al.*, 1965).

Higher alcohols are associated with a desirable full flavour and aroma, (Pollard *et al.*, 1966). The most important alcohol in any fermented beverage is of course ethanol. It is also the major volatile component in the product, giving body and viscosity by altering the acid-sugar balance (Rapp and Mandery, 1986). Ethanol also reduces apparent acidity, making ciders appear less sharp, increases sweetness and has an overall smoothing effect on

the other flavour components. It masks the otherwise overpowering aroma of butanols, pentanols and hexanols (Williams, 1972). It has a slightly fruity odour, which contributes to the overall aroma, but this is usually camouflaged by its interaction with other aroma constituents (Williams, 1972; Williams and Rosser, 1981). Ethanol is therefore largely responsible for the fermented flavour of the beverage (Pollard *et al.*, 1965) and is said to make a poor quality drink more palatable (Williams and Rosser, 1981).

Iso-butanol is present in high concentrations in apple juice and persists throughout the cider making. It enhances the sweetness and the fruity, scented flavours (Beech, 1972b; Beech and Carr, 1977). 3-Methylbutanol may also have important synergistic effects on flavour (Williams, 1974). During fermentation, higher alcohols dominate all other compounds, with the major concentration changes occurring with *iso*-butanol, 2-phenylethanol and the pentanols (Schreier *et al.*, 1978). After ethanol, *iso*-amyl alcohol is the main alcohol synthesised during fermentation by yeast; it may comprise 40-70 % of the total higher alcohol fraction during storage. There is also an increase in n-propanol and n-butanol and a decrease in 2-phenylethanol and hexanol.

Ethanol also has an inhibitory effect on malo-lactic bacteria and is generally accepted as the principle inhibitor of bacterial growth. Malo-lactic fermentation is delayed in ethanol concentration from 10% to 14%, but not necessarily prevented (Kunkee, 1967), as ethanol concentrations of up to 15% (v/v) can be tolerated by most lactobacilli (Calo *et al*, 1991). Resistance to ethanol varies from strain to strain (Britz and Tracey, 1990). The extent to which ethanol affects this secondary fermentation is conditional to the presence or absence of nutrients (Tracey and van Rooyen, 1988).

Esters

Esters make up the majority of non-polar volatile compounds in cider and other alcoholic beverages. Most are synthesised during fermentation, although small quantities are present in the juice, increasing 200-fold during fermentation (Beech and Carr, 1977). Principle apple juice esters are ethyl-2-methylbutyrate and hexyl acetate (Williams and Tucknott, 1971; Williams and Tucknott, 1978). Some fruit esters, in particular acetates, are degraded at the outset of fermentation and thus trace amounts are found in the end product (Schreier *et al.*, 1978). Esters derived from benzoic and 2-phenylacetic acids are the only aromatic

esters that have been determined in cider, ethyl dodecanoate is the only hydroxy ester and the butyl succinates are the only dibasic acid esters (Williams and Tucknott, 1978). Ethyl acetate is the primary ester produced. Fatty acid esters comprise the largest group of aroma compounds synthesised by yeasts. Hexanoic, octanoic, decanoic and dodecanoic acid esters increase the most during the fermentation, corresponding to the decrease in sugar concentration (Schreier *et al.*, 1978) and increase in ethanol concentration (Sakuma *et al.*, 1990; Rapp and Mandery, 1986).

Three reaction types have been suggested for the formation of esters: Reversal of hydrolysis, participation of acyl CoA and exchange reaction involving acyl CoA (Schreier, 1979). Reversal of hydrolysis, i.e. condensation, requires no acyl coenzyme A and this mechanism is utilised by non-brewing yeasts (Peddie, 1990). The reverse action of esterase enzymes can bring about the synthesis of esters, according to the reaction:

$$R^1OH + RCOOH \leftrightarrow RCOOR^1 + H_2O$$

Inter-esterification of acids and alcohols result in the synthesis of esters within the yeast cell, which diffuse into their surroundings. Ester levels in cider however, are too high to be synthesised wholly by this condensation reaction. Esters in cider arise from the alcoholysis of acyl CoA compounds. These are formed from keto acids as by-products of amino acid synthesis or from the leakage of products in the synthesis of higher fatty acids (Williams and Tucknott, 1978).

Ester synthesis is very low at the beginning of fermentation due to the high metabolic demand for acyl CoA. An equilibrium between the requirement for fatty acid, lipid and amino acid synthesis and ester production for this coenzyme is reached after about eight hours (Peddie, 1990). A peak in acyl CoA levels occurs as fatty acid and sterol synthesis ceases. This is followed by a second, short-lived induction of ester formation contributing significantly to the overall concentration of esters in the end product at the mid point of fermentation.

Acetate esters are synthesised by alcohol acetyl transferase, AAT. Substrates for this enzyme are alcohol and acetyl coenzyme A (CoASH) (Peddie, 1990):

$$RCOOH + ATP + CoASH \rightarrow RCO\text{-}CoA + AMP + PP_i + H_20$$

$$RCO\text{-}SCoA + R^1OH \rightarrow RCOOR^1 + CoASH$$

The other esters are synthesised by their corresponding fatty acyl coenzyme A compounds. If several such coenzymes are present, competitive inhibition may occur, thus inhibiting the esterification of individual activated compounds. Other inhibitors include *iso*-valeric and *iso*-butyric acid esters (Peddie, 1990).

After fermentation, esters are generally hydrolysed until their concentration approaches equilibrium with their component acid and alcohol, except when either component is in excess, due to microbial action (Beech and Carr, 1977). Ethyl esters hydrolyse slowly due to the high level of ethanol in the cider. Lower fatty acid esters (ethyl butanoate, propyl acetate, 3-methylbutyl acetate, hexyl acetate, ethyl octanoate and ethyl decanoate) decrease during storage, while mixed esters, such as 3-methylbutyl-3-methyl butanoate, ethyl lactate and diethyl succinate, increase (Williams, 1989).

These secondary products of fermentation usually have a fruity aroma, with a low odour threshold (Peddie, 1990). As the number of carbon atoms in the fatty acid chain increases, the corresponding ester imparts a softer aroma , tending towards a soapy/stearic odour (Rapp and Mandery, 1986).

Volatile Acids

Apple juice contains many volatile acids including formic, acetic, propionic, butyric and octanoic acids. During fermentation, medium chain fatty acids, such as hexanoic, octanoic, decanoic and dodecanoic are synthesised by yeasts and are excreted into the cider (Whiting, 1976). These fermentation by-products are toxic to yeasts and can completely inhibit their growth in the presence of ethanol (Munoz and Ingledew, 1989).

In cider, the following acids occur in decreasing order of concentration: butyric, hexanoic, octanoic, propionic, *iso*-butyric, 2-methyl and 3-methyl butyric acids.

Acetic acid is the main volatile fatty acid in alcoholic beverages. *Kloeckera*, *Pichia* and *Brettanomyces* species may form acetic acid during the initial stages of fermentation, but later some is metabolised (Whiting, 1976). Acetic acid bacteria are well known for their ability to spoil wines and ciders by the oxidation of ethanol to acetic acid.

Aliphatic and aromatic acids are considered to remain static or increase through lactic acid bacterial metabolism during the storage period. Acetic acid is one product of the fermentation of malic acid by heterofermentative lactic acid bacteria using the pentose phosphate pathway. These heterofermentative rods and cocci may also produce acetic acid from the fermentation of citric, pyruvic, quinic and shikimic acids (Beech, 1972b; Beech and Carr, 1977; Kunkee, 1967; Zúñiga *et al*, 1993).

Carbonyls

Carbonyl compounds are of great importance as aroma compounds because they have low sensory threshold values. Lactic acid bacteria are well known for their ability to produce flavour compounds, such as acetaldehyde, diacetyl and acetoin. These are of considerable importance to the flavour profile of wine and their production is closely associated with the growth and activities of micro-organisms (Davis *et al*, 1985).

Aldehydes, other than acetaldehyde, are intermediates in the formation of higher alcohols, through the decarboxylation of α-keto acids (Suomalainen and Lehtonen, 1979; Rapp and Mandery, 1986; Rodriguez *et al.*, 1990). Acetaldehyde, however, is produced as an intermediary product (of yeast metabolism) from pyruvate, hence its production is dependent on those parameters affecting ethanol synthesis. It has a dry, choking aroma (Williams, 1982).

Ketones, diketones and hydroxyketones are also formed during fermentation. 2,3-butanedione (diacetyl) and 3-hydroxybutane-2-one (acetoin) are the most commonly found ketones and are associated with ciders exposed to aerobic fermentation (Williams and Tucknott, 1971). Small amounts of diacetyl are produced by yeasts by the spontaneous decarboxylation of α-acetolactic acid and α-aceto-α-hydroxybutyric acid (Suomalainen and Lehtonen, 1979) during alcoholic fermentation; they may be metabolised through reduction to 2,3-butanediol (Henick-Kling *et al.*, 1993). Diacetyl and acetoin increase after malo-

lactic fermentation, particularly when lactobacilli are present (Crapisi *et al*, 1987b). The formation of acetoin is due to the reaction of two molecules of pyruvic acid to give α-acetolactic acid and carbon dioxide. α-Acetolactic acid is then decarboxylated to acetoin, which can then be reduced to butylene glycol. Diacetyl is an oxidation product of acetoin (Kunkee, 1967; Shimazu *et al*, 1985). A high concentration of intracellular pyruvate, or an additional source, such as citrate, is required for diacetyl and acetoin synthesis by lactic acid bacteria, as most of the pyruvate is converted to lactate to regenerate NAD (Benito de Cardenas et al., 1991a; Nuraida and Owens, 1992; Tsau *et al*, 1992). Heterofermentative cocci produce lactate and acetoin from citrate. Acetic acid and diacetyl are also formed. Generally, homofermentative bacteria produce more diacetyl than heterofermentative bacteria (Kunkee, 1967; Davis *et al*, 1985). The concentration of diacetyl may decline during later stages of storage (Davis *et al*, 1985).

At high concentrations, diacetyl imparts a buttery aroma to wines and at lower concentrations it may contribute to the nutty, yeasty and caramel aromas often described (Henick-Kling *et al.*, 1993). This applies to cider as well. It is present in most wines and ciders that have undergone malo-lactic fermentation (Radler, 1992). Organoleptic thresholds for diacetyl and acetoin have been reported in wine between 2 and 14 mg/l (Henick-Kling *et al.*, 1993) although a diacetyl concentration between 1 and 4 mg/l is considered to add complexity to wine; above this concentration, diacetyl is overpowering (Kunkee, 1974; Davis *et al*, 1985; Rodriguez *et al.*, 1990). In cider, acetoin has a threshold of 0.15 mg/l and diacetyl has a threshold as low as 0.001 mg/l (Durr, 1986).

Acetals

Acetal formation occurs primarily during storage. They are formed from the interaction of higher alcohols and aldehydes produced in the fermentation. The pH of cider suggests a chemical equilibration as opposed to enzymic activity (Williams and Rosser, 1981), but some compounds are artefacts of the extraction and concentration procedure (Yurchenko *et al.*, 1974). Cyclic acetals have a pungent, oily, green aroma whereas aliphatic acetals have a fruity, sharp, green aroma (Williams, 1989; Williams and Rosser, 1981; Hubert *et al.*, 1990). Williams (1982) suggested that these compounds impart little character to the product.

Sulphur Components

Sulphur compounds have low odour thresholds and often have a detrimental effect on flavour. They are produced in the autolysis of sulphur-containing amino acids, such as cysteine and methionine. Hydrogen sulphide is formed during fermentation (Suomalainen and Lehtonen, 1979).

Many volatile sulphur compounds are very flavour active, with low odour thresholds, even levels below 1 μg/l contributing to both aroma and taste (Williams, 1982). Typically, vegetable-like flavours are described, which impart undesirable characteristics to the product when present in excess (Walker and Simpson, 1993). Compounds present initially in apple juice are found to be lost to some extent during fermentation, as a result of purging by carbon dioxide. There are several sulphur containing compounds in cider, the formation of which are closely linked with yeast metabolism (Jarvis, 1993).

Lactones

Lactones impart an aroma associated with high quality wines (Hubert *et al.*, 1990). δ-nonalactone, δ-decalactone and τ-butyrolactone are examples of lactones found in cider. Biochemically, lactones are thought to be derived from glutamic acid via 4-ketobutyric acid, head and tail condensation of keto acids giving rise to various acyl lactones (Williams, 1982). Free hydroxy acids spontaneously undergo ring closure, or are converted by enzymes to lactones. Yeasts and moulds may possess such enzymes (Law and Wigmore, 1982).

Furans

The presence of furans has been identified in cider (Farris *et al.*, 1991; Yurchenko *et al.*, 1974) for example, furfural and dihydrobenzofurane. Their formation is thought to be derived from carbohydrate sources that have been heated and/or stored for a long duration (Williams and Rosser, 1981).

2.2 CIDER MICROBIOLOGY

2.2.1 Yeast

Ripe fruit harbours *Aureobasidium pullulans*, species of *Saccharomyces, Rhodotorula, Torulopsis, Candida* and *Kloeckera apiculata* (Beech, 1972a). The fungal population of apples is most frequently a combination of penicillia and aspergilli (Beech, 1972b). When the fruit is pressed, it acquires an additional microflora whose complexity varies with the degree of cleanliness of the milling and pressing equipment (Beech, 1972b).

The development of both yeast and bacteria in naturally fermented cider has been studied in French (Salih *et al.*, 1988) and Spanish cider (Salih *et al.*, 1990). Yeast show a proliferation phase, without a lag phase, followed by a quasi stationary phase before a long decline phase (Salih *et al.*, 1988). During this decline phase, alcohol production still continues (Salih *et al.*, 1988).

Saccharomyces strains grow rapidly in apple juice, dominating fermentation until the end of alcohol production (Salih *et al.*, 1988). In some cases, the initial yeast population is represented by apiculate yeasts, *Kloeckera apiculata* and *Hansenula anomala*, before being replaced by *Saccharomyces* species towards the middle of the fermentation (Salih *et al*, 1988). These apiculate yeast are considered to be responsible for ethyl acetate and butyrate overtones (Beech, 1993b). Some yeasts, in the genera *Saccharomyces, Debaryomyces, Torulopsis, Candida* and *Pichia* are able to produce killer factors (Ribéreau-Gayon, 1985). This can cause fermentation difficulties. Yeast in cider-making has recently been comprehensively reviewed by Beech (1993b).

The principle aroma fraction of alcoholic beverages is formed during yeast fermentation (Suomalainen and Lehtonen, 1979). Although different strains of yeasts produce qualitatively the same aroma compounds the quantitative differences can be substantial; for example, *Sacch. cerevisiae* produces significantly more *iso*-amyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate than does *Sacch. uvarum* (Suomalainen and Lehtonen, 1979). At the end of fermentation, the cider is racked off the lees and left in storage. Storage of cider occurs in very large vats, until sale, minimising deleterious changes, which

can occur if a surface flora of aerobic yeast (*Pichia* and *Candi*da spp.) and acetic acid bacteria is allowed to develop during storage (Beech, 1993b).

2.2.2 Acetic Acid Bacteria

Most common spoilage bacteria of alcoholic products are members of the family Acetobacteraceae, consisting of the two genera *Acetobacter* and *Gluconobacter* (Kösebalaban and Özilgen, 1992). Acetic acid bacteria isolated from apple juice include *Acetobacter xylinum, A. rancens, A. oxydans, A. mesoxydans, A. aceti* and *Acetomonas oxydans* (Beech, 1972b). Acetic acid bacteria are introduced into cider from the fruit and processing equipment. They are suppressed as soon as fermentation commences, although they are able to grow again once fermentation has ceased and conditions become slightly more aerobic (Passmore and Carr, 1975).

Approximately 38% *Gluconobacter* spp., 50% *A. rancens*, 6% *A. mesoxydans* and 6% *A. xylinum* were found in fermenting juices and dry ciders by Passmore and Carr (1975). In cider from the Asturian province of Spain, 38% of isolates were strains of *G. oxydans*, 61% *A. acetii*, 0.8% *A. liquefaciens* and 0.2% *A. pasteurianus* (Salih and Suarez Diaz, 1990).

Acid tolerance and the ability of acetic acid bacteria to utilise ethanol as an energy source have enabled these micro-organisms to survive in quite exclusive habitats, especially in fruit based beverage production (Passmore and Carr, 1975). They are able to metabolise ethanol to acetic acid, thereby accelerating yeast death (Gilliland and Lacey, 1966; Kaneko and Yamamoto, 1966) and spoiling the beverage (Drysdale and Fleet, 1989). This phenomenon is accentuated at high temperature and pH (Joyeux *et al.*, 1984). It has been implied by Kösebalaban and Özilgen (1992) that some of the micro-organisms in the population become acid tolerant in the death phase. Biochemical characteristics of acetic acid bacteria, such as cellulose formation, gluconic acid production, ketogenesis, pigmentation and loss of ethanol oxidising ability, are variable (Kösebalaban and Özilgen, 1992).

Gluconobacter oxydans usually converts sugars to acetic and lactic acids via the hexose monophosphate pathway, but under conditions of low pH and high glucose concentration,

this pathway is repressed and glucose is converted to gluconic acid (Drysdale and Fleet, 1989). Strains of *Acetobacter* have a weak ability to metabolise sugars, albeit by the same mechanism.

Acetic acid bacteria have always been reported to be strictly aerobic, considered unable to survive in wines and ciders except at the surface in contact with the air. Recent findings, however, suggest that acetic acid bacteria are capable of surviving under anaerobic and microaerophilic conditions (Kösebalaban and Özilgen, 1992). Thus, there is a permanent presence of acetic acid bacteria at every stage of the process (Beech, 1972b). These micro-organisms rapidly multiply when exposed to air, however briefly, unfavourably affecting quality (Kösebalaban and Özilgen, 1992). Aeration during racking and transfer as well as diffusion of air across the vat walls are means by which these micro-organisms are supplied with air (Joyeux *et al.*, 1984; Ribéreau-Gayon, 1985; Salih and Suarez Diaz, 1990). Acetic acid bacteria are responsible for the small increases in acetic acid sometimes observed, even during storage under the best of conditions (Joyeux *et al.*, 1984). There is a successive change in the acetic acid bacteria population during fermentation and storage, starting with *Gluc. oxydans*. Then *A. pasteurianus* and *A. acetii* become dominant. The latter bacteria species can survive during storage as a quantity of oxygen penetrating the wooden vats prevents complete destruction of the population (Joyeux *et al.*, 1984; Drysdale and Fleet, 1989).

Gluconobacter oxydans, *Acetobacter pasteurianus* and *A. aceti* have an ability to grow in conjunction with *Sacch. cerevisiae* during alcoholic fermentation, interfering with its progress and commonly causing stuck or incomplete fermentation (Drysdale and Fleet, 1989). These bacteria are ubiquitous during every stage of wine making. *Gluc. oxydans* was reported to be dominant during alcoholic fermentation and was subsequently replaced by *A. aceti* and *A. pasteurianus* (Joyeux *et al.*, 1984; Salih and Suarez Diaz, 1990). Major spoilage is reported to occur during the extended presence of micro-organisms in the stationary and death phases, as opposed to the growth phase as acid formation is non-growth associated (Kösebalaban and Özilgen, 1992). The development of acetic acid bacteria cause changes in composition such as the production of acetaldehyde and ethyl acetate which are detrimental to product quality. They oxidise glycerol to dihydroxyacetone and some strains are able to metabolise organic acids (Drysdale and

Fleet, 1989). Salih and Suarez Diaz (1990) reported that the production of acetic acid was due not only to the action of acetic acid bacteria, but equally to the presence of lactic acid bacteria.

Gilliland and Lacey (1966) reported that a particular strain of *Acetobacter*, isolated from bottled beer, either inhibited yeast growth and fermentation or even killed *Saccharomyces cerevisiae* in an anaerobic environment. They demonstrated that the critical factor for inhibition or death was the ratio of their strain of *Acetobacter* to yeast cells. The *Acetobacter* strain, closely related to *A. rancens*, was also reported to kill lactobacilli under anaerobic conditions.

2.2.3 Lactic Acid Bacteria

Characterisation

Lactic acid bacteria are an extensive group of Gram positive, catalase negative, asporogenous, non-motile, anaerobic or microaerophilic lactic acid producing micro-organisms. They include the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus*. They can be divided into two physiological groups, depending on their hexose pathway: homofermentative or heterofermentative. Homofermentative lactic acid bacteria degrade hexoses via glycolysis (Embden-Meyerhof-Parnas pathway), producing lactic acid as the major end product, whereas the heterofermentative lactic acid bacteria use the pentose phosphate pathway and yield lactic acid, carbon dioxide, acetic acid and/or ethanol (Kunkee, 1967; Beech, 1972b; Beech and Carr, 1977; Zúñiga *et al*, 1993). The lactic acid bacteria found in cider can be further subdivided, on the basis of cell morphology, into rods and cocci. *Pediococcus* (homofermentative) and *Leuconostoc* (heterofermentative) are the two genera of cocci, and the genus *Lactobacillus* contains both homo- and heterofermentative rods (Kunkee, 1967).

Lactic Acid Bacteria Found in Cider

The organisms reported as occurring most frequently in English cider are non-slime forming strains of *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, the most commonly isolated homofermentative rod (Beech, 1972b), and *Lact. collinoides* the most commonly isolated heterofermentative rod (Beech, 1972a; Carr and Davies, 1972).

Heterofermentative species are more common than homofermentative species, which occur only sporadically (Carr, 1959). The only homofermentative coccus, *Pediococcus cerevisiae*, was found to occur rarely (Beech, 1972b). Similar types of lactic acid bacteria are found in wine (Watson, 1986).

The occurrence of these genera in English cider were found to be comparable to those in Bordeaux wines: 20% heterofermentative rods, 57% heterofermentative cocci, 16% homofermentative rods and 7% homofermentative cocci (Beech, 1972b). In a Spanish cider, Salih and his colleagues (1990) reported 86% *Leuconostoc* spp., 13% homofermentative *Lactobacillus* (*Lact. brevis*) and 1% *Pediococcus* spp. In a French cider, 48% lactic acid bacteria isolates were identified as *Leuc. oenos* (Salih *et al*, 1988).

Occurrence of Lactic Acid Bacteria

Lactic acid bacteria grow in several phases during the production of ciders and wines and different species may be associated with each phase (Wibowo *et al.*, 1986). They are rarely found on the fruit, but rapidly become part of the indigenous flora once in the factory, as a result of infection by milling and pressing equipment (Beech, 1972b; Lafon-Lafourcade and Ribereau-Gayon, 1984).

Lactobacilli are generally found in fermenting cider, which is probably a reflection of their temperature responses. It is possible that elevated fermentation temperatures encourage the growth of these bacteria (Beech and Carr, 1977). *Leuconostoc* spp. are more likely to be encountered after fermentation, in the cooler conditions of storage (Beech and Carr, 1977).

Growth

Yeasts and bacteria coexist in apple juice during fermentation, but the conditions are initially more favourable to the yeasts. Interrelationships between yeasts and bacteria may have a significant influence on the activity of each strain. There is a consistent decrease in both number and type of lactic acid bacteria during alcoholic fermentation, possibly due to the lack of alcohol tolerance of initial strains (Lafon-Lafourcade and Ribereau-Gayon, 1984) and initial low levels and high phenolic compound content (Salih *et al*, 1988). Towards the end of fermentation, the lactic acid bacteria grow more vigorously in the anaerobic conditions created by the yeasts as low concentrations of carbon dioxide

stimulate lactic acid bacteria (Carr, 1959; Leroi and Pidoux, 1993a). The bacteria utilise the remaining nutrients and recycle nitrogenous compounds released by the yeasts (Passmore and Haggett, 1973).

Inhibition of bacterial growth is most probably due to the presence of yeast metabolites such as ethanol and sulphur dioxide and/or the removal of essential nutrients or growth factors (King and Beelman, 1986; Salih *et al*, 1990). The transition from lag to log phase of bacterial growth, however, coincides with the death phase of yeast and may result in the return of essential nutrients to the medium as a result of yeast autolysis (King and Beelman, 1986).

Generally, yeast and bacteria compete for nutrients such as fructose, glucose and sucrose. Since the bacteria grow more slowly than the yeasts, they must also be able to utilise components of the apple juice and cider not metabolised by the yeast. Under these conditions, specialised micro-organisms will be selected (Carr, 1959). Yeasts provide vitamins, amino acids and growth factors for the bacteria (Salih *et al.*, 1987; Leroi and Pidoux, 1993a) as these micro-organisms have exacting nutritional demands. These requirements play a very important role in controlling bacterial growth in fermenting ciders. The greatest growth of lactic acid bacteria can occur in the juice before the onset of fermentation and in fermented cider.

The most important characteristic of cider lactobacilli is their tolerance of very acid conditions. pH affects the metabolism of sugar and has a beneficial effect on the species, allowing growth to take place when other organisms are unable to grow (Britz and Tracey, 1990). Heterofermentative organisms are able to grow at pH values encountered in very acid ciders, whereas homofermentative organisms appear to be less well adapted to highly acid conditions (Carr, 1959). Indeed, *Pediococcus* spp. rarely grow below pH 3.5. They are most often found after malo-lactic fermentation when the pH has increased. Growth of pediococci at this stage is undesirable (Davis *et al.*, 1986).

Survival and growth of strains vary considerably in response to physico-chemical factors such as pH, ethanol and sulphur dioxide concentrations and temperature (Lafon-Lafourcade and Ribereau-Gayon, 1984; Wibowo *et al.*, 1988; Britz and Tracey, 1990). The

high concentrations of alcohol and acid, plus the presence of sulphur dioxide and the lack of nutrients make cider and wine a fairly hostile environment for micro-organisms (Kunkee, 1967).

Sulphur dioxide is strongly inhibitory to lactic acid bacteria, if not lethal. The addition of up to 50 mg/l of sulphur dioxide does not reduce the initial lactic acid bacteria population, although 100 mg/l reduces it around ten-fold (Lafon-Lafourcade and Ribereau-Gayon, 1984). The synergistic effect of sulphur dioxide with pH is very inhibitory to lactic acid bacteria. A decrease in pH shifts the equilibrium of the reaction in the direction of molecular sulphur dioxide, which is more toxic to bacterial growth than the other two ionic forms. The higher the sulphur dioxide concentration and the lower the pH, the greater the inhibitory effect on growth (Britz and Tracey, 1990). Of the unbound of sulphur dioxide, only sulphurous acid is active on the metabolism of these bacteria, probably by oxidation of the disulphide group of proteins (Kunkee, 1967). Lactic acid bacteria metabolise some sulphur binding compounds, freeing sulphur dioxide, thus increasing the inhibitory effect (Kunkee, 1967; Radler, 1992). Heterofermentative rods are very sensitive to sulphur dioxide (Carr and Davies, 1972), although Davis and colleagues (1986) found that some species of *Lactobacillus*, presumably homofermentative bacteria, were more tolerant of sulphur dioxide than many other lactic acid bacteria. Heterofermentative *Leuconostoc* spp. are most often found in fermented cider. In those ciders where the use of fairly high concentrations of sulphur dioxide is usual, the predominant bacteria were *Leuconostoc* spp. while *Lactobacillus* spp. predominated when low sulphur dioxide levels were present (Beech and Carr, 1977). Lactobacilli have the greatest tolerance of bound sulphur dioxide, leuconostocs intermediate and pediococci the least (Beech, 1993a). Indeed, these bacteria remain viable in wine during storage, exhibiting no tendency for further growth and showing only slow, progressive decline in viability over 200 days (Lafon-Lafourcade and Ribereau-Gayon, 1984).

Malo-lactic Fermentation

Malo-lactic fermentation, its effects and control, has been investigated primarily in wines. Although wine and cider microbiology and biochemistry have many parallels, it can not be assumed that they are identical. It is important, therefore, that malo-lactic fermentation in cider is studied and comparisons made with wine malo-lactic fermentation.

Yeast fermentation of fruit juices, such as apple juice is frequently accompanied, or followed by, a second fermentation carried out by bacteria, universally known as malo-lactic fermentation (Beech, 1972b). It is the conversion of malic acid to lactic acid and carbon dioxide. The acidity of the beverage is halved, with the loss of one of the two carbonyl groups from malic acid (Beech, 1972b). Reduction in acidity can vary from between 0.1% to 0.3% and the pH may rise by 0.1 to 0.3 of a unit (Davis *et al*, 1985). During malo-lactic fermentation, lactic acid bacteria develop to a cell density of 10^7 to 10^8 cells ml^{-1} (Kunkee, 1974; Davis *et al*, 1985). A temperature of 15°C and above encourages this change (Beech and Carr, 1977). Warm upper layers allow malo-lactic fermentation to take place, whereas no activity may take place at lower, cooler levels (Beech and Carr, 1977).

Malo-lactic fermentation may occur spontaneously during or after alcoholic fermentation (Henick-Kling *et al.*, 1993). French and English cider normally undergoes malate conversion after the alcoholic fermentation and during storage as lactic acid bacteria populations are smaller than those in Spanish cider and have to increase in number before commencing malo-lactic fermentation (Salih *et al*, 1990). In wine, the lag phase between completion of alcoholic fermentation and commencement of malo-lactic fermentation varies from 0 to 50 days. The length of this phase depends on vinification practices and wine properties. Low pH, high sulphur dioxide and high ethanol concentrations lengthen this lag phase (Davis *et al.*, 1986).

Malo-lactic fermentation is encouraged in wines for the purposes of deacidification, microbial stabilisation and/or modification of flavour and aroma (Kunkee, 1974; Lafon-Lafourcade and Ribereau-Gayon, 1984; Watson, 1986; Rodriguez *et al.*, 1990). The benefits and disadvantages of malo-lactic fermentation depend upon region, grape variety, wine composition, wine making techniques and the wine maker (Davis *et al*, 1985). Depending on the type of wine, malo-lactic fermentation may or may not be desired and promoted (Radler, 1992).

A better balanced wine from highly acidic wine may be produced, since the sharp taste is replaced by a more mellow, smooth flavour (Henick-Kling *et al.*, 1993). Those wines produced in cooler climates, such as in Germany, France and eastern United States do

indeed benefit (Kunkee, 1974; Davis *et al*, 1985; Watson, 1986). Wines from warmer regions, such as those produced in California, South Africa and Australia do not benefit from this reduction in acidity, resulting in flat, insipid wines (Kunkee, 1967; Davis *et al*, 1985; Dicks and van Vuuren, 1988). Lactic acid bacteria not only deacidify wine, but can also modify fruity and vegetative aromas and improve mouthfeel, length of aftertaste and increase fullness (Henick-Kling *et al.*, 1993). The production of some amount of polysaccharide may contribute to mouthfeel and body. More likely, this improved mouthfeel is due to flavour enhancing substances that have not, as yet, been recognised (Henick-Kling *et al.*, 1993).

It is generally assumed that a wine is stable to further bacterial growth on completion of malic acid degradation (Crapisi *et al*, 1987a; Crapisi *et al*, 1987b; Dicks and van Vuuren, 1988; Rodriguez *et al.*, 1990; Velázquez, *et al*, 1991). Wine, however, has been found to support a succession of lactic acid bacteria. Thus, stability and quality of wines after malo-lactic fermentation is more dependent on subsequent processing, than on the effect of malo-lactic fermentation (Davis *et al*, 1985; Watson, 1986; Henick-Kling *et al.*, 1993). Kunkee (1974) and later Radler (1992) reported no instances where a second bacterial fermentation had occurred once malo-lactic fermentation was completed, unless additions had been made to wines.

The efficiency with which strains conduct malo-lactic fermentation in the same wine varies and single strains exhibit variable performance in different wines (Wibowo *et al.*, 1988). *Leuconostoc paramesenteroides* and *Lactobacillus plantarum* degrade L-malic acid most rapidly. *Leuc. oenos* and *Lact. fructivorans* are a little slower (Pardo and Zuñiga, 1992). Strain of bacteria is also important as the rate of breakdown of malate is generally greater with rods than cocci (Beech, 1972b). There is no difference in malo-lactic fermentation when inoculated with *Leuc. oenos* strains at the beginning, middle or end of fermentation, whereas there are large differences when *Lactobacillus* strains are used (Tracey and van Rooyen, 1988).

During malo-lactic fermentation, lactic acid is the most abundant end product, formed from sugars, malic acid and citric acid. Depending on the organism, D-lactic acid, L-lactic acid or a mixture of D- and L- isomers may be formed (Kunkee, 1967). *Leuconostoc* species

form D-lactic acid from glucose, but L-lactic acid from L-malic acid (Kunkee, 1974; Lafon-Lafourcade and Ribereau-Gayon, 1984).

Bacteria also metabolise substrates, such as sugars, amino acids, organic acids and glycerol. This may lead to spoilage, particularly the degradation of sugars which leads to a "lactic flavour". This phenomenon is known in France as `piqûre lactique' when the concentration of D-lactic acid is greater than 2.2 mM (Salih *et al*, 1990). It does not, however, appear in unpleasant quantities when a cider low in phenolics is pitched with low levels of both lactic acid bacteria and yeast (Salih *et al*, 1987). Delaying the onset of malo-lactic fermentation until all the sugars have been fermented by yeast is desirable to prevent D-lactic acid production (Lafon-Lafourcade and Ribereau-Gayon, 1984). Other forms of bacterial spoilage may involve the production of acetic acid and other metabolic by-products (Beech, 1972b; Salih *et al*, 1990).

Rodriguez and her colleagues (1990) commented that extended lees contact and fermentation in wood at warm temperatures may be as effective as malo-lactic fermentation in producing complexity in wines. They reported that stirring of lees was practised in Burgundy as a means of achieving similar complexity to that produced by malo-lactic fermentation .

Lactic acid bacteria are well known for their ability to produce flavour compounds, such as acetaldehyde, acetic acid, ethanol, diacetyl, acetoin and 2,3-butanediol. The last three are of considerable importance to the flavour profile of wine and their production is closely associated with the growth and activities of micro-organisms (Davis *et al*, 1985). Generally, homofermentative bacteria produce more diacetyl than heterofermentative bacteria (Kunkee, 1967; Davis *et al*, 1985). The concentration of diacetyl may decline during later stages of storage (Davis *et al*, 1985).

The use of modern wine making techniques allows the production of wines with and without malo-lactic fermentation, and with the availability of selected pure cultures for induction, the wine maker now has good control over the quality of malo-lactic fermentation (Henick-Kling *et al.*, 1993). It is often, however, difficult to promote or control malo-lactic fermentation in wines (Crapisi *et al*, 1987a). There are several options

available: selection of conditions encouraging natural malo-lactic flora growth; induction of malo-lactic fermentation by inoculation with wine already undergoing malo-lactic fermentation; induction of malo-lactic fermentation by inoculation with either laboratory prepared or commercial strains of lactic acid bacteria; passing wine over immobilised lactic acid bacteria; use of yeast to degrade malic acid; use of carbonic maceration for partial degradation of malic acid prior to natural malo-lactic fermentation; inhibition of malo-lactic fermentation (Davis *et al*, 1985).

The ways to induce or promote malo-lactic fermentation are well documented: long contact with lees, low sulphur dioxide, storage temperature of 18°-22°C and pH above 3.2 (Kunkee, 1974; Radler, 1992). Inoculation of a wine with lactic acid bacteria at a cell density in excess of 10^6 cfu/ml significantly enhances the probability of obtaining a rapid and complete malo-lactic fermentation. Under these conditions, the cells act as a direct source of the malic degrading enzyme(s), so that malo-lactic fermentation is divorced from the need for cell growth, and is, as a consequence, free from any adverse influences of wine properties on growth (Beech, 1972a; Beech, 1972b; Salih et al., 1987; Salih et al., 1988; Wibowo *et al.*, 1988; Salih et al., 1990).

Conservation techniques, such as racking off the lees, at the end of fermentation to prevent autolysis and subsequent release of micro-nutrients, lowering temperature to below 15°C, clarification, increasing sulphur dioxide content and lowering acid levels are major factors in rendering a wine stable (Kunkee, 1974; Davis et al., 1985; Rodriguez *et al.*, 1990). The synergistic effect of these parameters has much greater effect on bacterial growth than each parameter individually (Britz and Tracey, 1990).

2.2.4 Other Bacteria

Goverd and his colleagues (1979) suggested that coliforms may form part of the normal bacterial flora of orchards and cider factories. Use of water flumes to transport fruit to the mill increases numbers of coliforms in the juice. Fermentation decreases the survival ability of coliforms, due partly to the presence of ethanol and partly to the changes in nutritional and physiological conditions.

2.3 SUMMARY

- As pomology and fermentation technology have been the main concerns in cider research, knowledge of microbial and chemical changes of cider during storage is limited. Wine research can only provide guidelines for cider maturation changes.

- A vast amount of information has been amassed on flavour compounds in cider, in particular the volatile components. Factors affecting flavour compound synthesis and removal are, however inadequately understood, especially during maturation.

- Microbiological changes in cider produced by natural fermentation are relatively well documented, chiefly by French and Spanish cider researchers. Microbiological changes that occur after fermentation by pure yeast culture inoculation remain undefined.

- Although wine and cider microbiology and biochemistry have many parallels, it can not be assumed that they are identical. It is important therefore, that maturation changes in cider are studied.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 MICROBIOLOGICAL MEDIA

The media utilised for isolation and enumeration of micro-organisms found in cider, and for their subsequent maintenance, were recommended for use by the National Association of Cider Makers (NACM, 1989). Details of formulation and preparation of these media are in Appendix I.

3.1.1 Isolation and Enumeration Media

Lysine Agar

Lysine Agar is a complex synthetic medium for the isolation and enumeration of 'wild' or non-*Saccharomyces* yeast strains in pitching yeast cultures or fermenting juices. Species that grow readily on Lysine Agar include *Kloeckera*, *Hanseniaspora*, *Candida*, *Hansenula*, *Pichia*, *Saccharomycodes* and *Brettanomyces* (Morris and Eddy, 1957).

0.1 ml aliquots of cider, or a dilution thereof, were spread plated onto this agar. Inoculated plates were incubated aerobically at 28°C for 7 days.

Raka Ray Agar

Raka Ray Agar supports strong growth of lactic acid bacteria, owing to its nutritional characteristics. Yeast growth is suppressed by the addition of cycloheximide and the growth of Gram negative bacteria are suppressed by addition of phenyl ethanol. Anaerobic incubation inhibits growth of aerobic Gram positive organisms (Saha *et al.*, 1974).

These plates were utilised for spread plating cider, or dilutions thereof in 0.1 ml aliquots, or streaking out isolates for lactic acid bacteria purification and culture. In both cases, the plates were incubated anaerobically at 28°C for 7-10 days, until good growth had been achieved.

W.L. Differential Agar (WLD)

WLD is a selective medium that suppresses the growth of pitching yeast and most 'wild' yeast by the action of cycloheximide, and allows the growth of acetic acid bacteria (*Gluconobacter* spp. and *Acetobacter* spp.) and some Enterobacteriaceae (NACM, 1989).

Spread plates of 0.1 ml aliquots of cider, or a dilution thereof, were made using this agar. Inoculated plates were incubated aerobically at 28°C for 7 days.

W.L. Nutrient Agar

W.L. Nutrient Agar is recommended for determination of the microbiological flora in brewing and fermentation processes (NACM, 1989).

These plates were utilised for spread plating cider or dilutions thereof in 0.1 ml aliquots or streaking out isolates for yeast and acetic acid bacteria purification and culture. In both cases, the plates were incubated aerobically at 28°C for 5-7 days, until good growth had been achieved.

3.1.2 Maintenance Culture

Maintenance of pure cultures helps to stabilise the micro-organism's characteristics and ensures pure, active cultures are available for propagation and subsequent investigation.

Basal Broth (Bacteria)

Basal Broth is a defined non-selective growth medium utilised for liquid maintenance culture (Whittenbury, 1963).

Two loopfuls of pure bacterial isolate were aseptically inoculated into a 7 ml sterile Bijou containing 5 ml Basal Broth and incubated at 25°C for seven days. The cultures were refrigerated at 4°C until required.

MYGP Agar (Yeast)

Malt Extract, Yeast Extract, Glucose, Peptone (MYGP) agar is a non-selective medium used for the maintenance of yeast cultures in agar slopes (NACM, 1989).

A loopful of pure yeast isolate was aseptically streaked onto an agar slope and incubated at 25°C for 5-7 days until profuse growth was observed. The slopes were then refrigerated at 4°C until required.

3.2 CIDER FERMENTATION

Cider is fermented either from freshly pressed apple juice, during the harvesting season, or from an apple juice base derived from concentrated juice. Cider of consistent quality was required for maturation studies, thus, cider from concentrated apple juice base was primarily used. Cider from fresh apple juice was, however, utilised for investigating the effect of the presence of pulp particles on maturation flavour.

3.2.1 Formulation of Concentrate-Derived Apple Juice Base

Apple Juice Concentrate	84 ml
Glucose Syrup	118 ml
Thiamine Solution 0.1% (w/v)	0.22 ml
Ammonium Carbonate	0.1 g
Ammonium Sulphate	0.1 g
Sodium Metabisulphite	0.203 g
Distilled/deionised water	798 ml

Apple juice, in particular apple juice concentrate, is often deficient in nitrogenous compounds and thiamine (vitamin B1) which are significant yeast growth factors. Addition of these factors to the base encourages fermentation to proceed to completion.

3.2.2 Formulation of Fresh Apple Juice Base

Fresh Apple Juice	928.4 ml
Glucose	71.6 ml

Fresh apple juice was obtained ready sulphited (total SO_2 70 ppm).

3.2.3 Preparation and Fermentation of Apple Juice Bases

A glass fermenter of requisite capacity (2 l, 5 l or 10 l) was assembled with a gas inlet and outlet, each with gas permeable filters attached. The fermenter was sterilised by autoclaving at 121°C for 15 minutes, then charged with the sulphited apple juice base ingredients and stored at 5°C until inoculation.

100 ml aliquot of the apple juice base was aseptically transferred from the fermenter to a sterile baffle flask and inoculated with a generous loopful of yeast. The yeast strain used in all the fermentations was a commercial strain of *Saccharomyces cerevisiae* (AWY650R). This inoculum for the apple juice base was incubated at 25°C for two days.

Apple juice base was allowed to equilibrate to the fermentation temperature of 20°C in a constant temperature water bath. It was then inoculated with the previously grown yeast inoculum. Aerobic conditions were maintained for the first three days by passing filtered air into the apple juice base as fine bubbles, at a rate of 0.1 l/hr. This allowed for sufficient biomass formation to ferment the cider rapidly.

3.2.4 Analysis of Selected Fermentation Parameters

An ideal fermentation is one which ferments quickly to dryness. If conditions are not correct, the fermentation may be slow allowing off-flavours to develop. It is important, therefore, to monitor the progress of fermentation. Specific gravity of the broth is an indication of its sugar content, thus on the assumption that all the sugar is fermented to alcohol, potential alcohol content is calculated. A direct conversion cannot be applied as the juice also contains soluble, non-fermentable compounds that contribute to the specific gravity. Yeast counts and yeast viability establish the presence of a sufficient number of living yeast cells to effect a rapid fermentation.

Specific Gravity (S.G.)

Specific gravity measurements were performed using a 10 ml density bottle. The cider sample was centrifuged at 4000 rpm for 20 minutes and filtered through a 0.45 μm cellulose membrane filter.

Total and Viable Cell Counts

0.1 ml of well-mixed fermentation broth was aseptically removed from the fermenter and added to 4.9 ml 1/4 Strength Ringer's solution and mixed. 0.5 ml of this suspension was added to an equal volume of Methylene Blue stain and mixed. A small quantity of this suspension was transferred via a glass Pasteur pipette to the counting chamber of an improved Neubauer haemocytometer. After the cells had been allowed to settle for about three minutes, any yeast cells within the whole grid were counted (using the x 40 objective lens of the microscope) to give a total cell count. Non viable cells were also counted as they are differentiated from the living cells by penetration of Methylene Blue stain. The difference between the total and dead cell counts gave the viable cell count, which was then converted into % viability. The counting chamber was recharged twice more and an average total cell count and % viability was attained.

3.2.5 Cider Filtration

Unless otherwise stated, all cider prepared in the laboratory was microfiltered, using a sequential filtration system. At dryness, the cider was chilled at 5°C for 24 hours then aseptically filtered though a 0.45 µm, then a 0.2 µm Millipore membrane unit into autoclaved 2.5 l glass storage vessels.

3.3 CIDER MATURATION - LABORATORY SCALE

Maturation cider was stored at 15°C, unless otherwise stated, in a custom made, temperature controlled water bath. 2.5 l glass storage vessels were employed throughout laboratory scale cider maturation studies. Carbon dioxide was permitted to escape via an airlock (containing distilled water) fitted to each storage vessel, which also prevented oxygen penetration. Collection of samples from the sampling port was performed using a sterile needle and syringe to draw off the requisite volume of cider .

3.4 FLAVOUR ANALYSIS

3.4.1 Volatile Aroma Compound Analysis

Low molecular weight volatile compounds in cider, such as esters, alcohols, carbonyls and acids were extracted, concentrated, separated and analysed by gas chromatography at H P

Bulmer Ltd. Identification and calibration files were set up for selected compounds (Table 3.1). Several compounds were considered to be associated with apple and cider flavour, for example, hexyl acetate and ρ-ethyl phenol, while others were selected as potential maturation marker compounds, as reported in literature. Examples of these compounds are ethyl lactate, diethyl succinate and ethyl octanoate (Williams, 1989).

Sample Preparation

20 ml sample capacity ChemElut tubes, (Analytichem International) were used as solid phase separation columns.

A 20 ml aliquot of cider was introduced at the top of the tube followed by 1 ml heptan-3-one at 0.42 mg/l (internal standard) and eluted by gravity with two successive 20 ml aliquots of dichloromethane (Aldrich, UK), after a 15 minute resting period. The elution took about 45 minutes. The eluent, collected in round bottomed, 50 ml evaporation flasks, was concentrated to about 1 ml using a rotary evaporator in a 40°C waterbath. The final volume was adjusted under a stream of nitrogen to 50 µl. Concentration took about 10 minutes.

Apparatus and Column

The main aroma compounds were identified by a gas chromatograph, Unicam 610 series (ATI Unicam, Cambridge, UK) coupled with a FID. Data was handled by the Unicam 4880 chromato-integrator series (ATI Unicam, Cambridge, UK). The injections (0.02 µl) were performed by the Unicam 610 series automatic injector (ATI Unicam, Cambridge, U.K) into the injector held at 275°C onto a DBWAX 20M capillary column (Fisons Scientific Equipment, Leicestershire, U.K.), 50 m, i.d. 0.32 mm, stationary phase thickness 0.25 µ. Temperature programme maintained the oven at 60°C for 8 minutes, ramping at a rate of 2.5°C/min to 180°C and then at 4°C/min to 220°C for 8 minutes. Carrier gas, helium flowed at a rate of 1 ml/min.

Table 3.1 Flavour compounds and their associated aromas, analysed and identified by GC

COMPOUND	AROMA CHARACTERISTICS
ethyl-2-methyl butyrate	green, fruity, pungent, apple peel, cidery[2]
hexanal	fatty, green, grass, penetrating[1]
iso-amyl acetate	fruity, banana, sweet, fragrant[1]
2-methyl-1-butanol	choking, alcoholic, wine-like, fruity[2]
ethyl hexanoate	fruity, wine-like, apple, banana[1]
hexyl acetate	apple, cherry, pear, floral[1]
octanal	fatty, citrus, honey[1]
ethyl lactate	mild, ethereal, buttery[2]
hexanol	alcoholic, ethereal, medicinal[1]
nonanal	floral, citrus, orange, rose, fatty, waxy[1]
ethyl octanoate	fruity, wine-like, fragrant, apricot/banana[2]
undecanal	fatty, with orange and rose undertones[1]
ethyl decanoate	brandy, oily, fruity, grape[1]
ethyl benzoate	heavy, floral, fruity[1]
diethyl succinate	faint, pleasant[1], wine-like, ethereal[2]
phenylethyl acetate	sweet, honey, rose[1]
ethyl dodecanoate	oily, fatty, leafy[2]
2-phenyl ethanol	rose, honey, fragrant, floral[1]
heptanoic acid	rancid, sour, sweat-like, fatty[1]
4-ethyl guaiacol	spicy, medicinal, burnt toffee, phenolic[2]
octanoic acid	oily, fatty, rancid[1]
4-ethyl phenol	medicinal, phenolic, pungent[1]
4-ethyl catechol	[3]

[1] Aldrich, 1995. [2] Williams, 1974. [3] no aroma data found.

There was approximately 20% error associated with this analytical method, which had been assessed by using a standard solution and calculating recoveries. The greatest error was associated with the concentration stage, particularly when using the rotary evaporator.

3.4.2 Organic Acid Analysis

Low molecular weight organic acids were separated by high performance liquid chromatography at H P Bulmer Ltd, U.K..

Apparatus and Column

A high performance liquid chromatograph, Dionex 2000i (Dionex Corporation, Camberley, U.K.) was used with a CDMII Dionex conductivity detector. This apparatus was modified for use with a Gilson 221 (Gilson Medical Electronics, Villiers-le-Bel, France) sample changer and Gilson 401 dilutor. The sample loop was 20 µl. The column was a Dionex IonPac ICE-AS6 (250 x 9 mm i.d.). Data was handled by the Unicam 4880 chromato-integrator.

Chromatographic Procedure

Throughout the analysis, temperature compensation was maintained at -2 units and the background conductivity at approximately 13 µS. The latter was achieved with a micromembrane suppressor, using 0.4 mM heptafluorobutyric acid (Aldrich, U.K.) as the eluent and 0.4 mM tetrabutylammonium hydroxide (Fluka Chemicals Ltd., U.K.) as the regenerant. Both were made up with distilled, deionised water and vacuum filtered. The flow rate was 1 ml/min at a pump pressure of approximately 300 psi. Run time was 20 minutes.

Sample Preparation

Samples were filtered with a Titan 2 µm pore nylon membrane HPLC syringe filter (Scientific Resources Inc., New Jersey, USA) into 2 ml crimp top vials.

3.4.3 Sugar Analysis

Selected mono- and di-saccharides were separated by high performance liquid chromatography, at H.P.Bulmer Ltd., U.K.

Apparatus and Column

A high performance liquid chromatograph, Dionex 4000 (Dionex Corporation, Camberley, U.K.) was used with a pulsed amperometric detector and Dionex AS3500 autosampler.

Injection volume was 5 µl. The column was a Dionex CarboPac PA1 (250 x 4 mm i.d.). Data was handled by a Dionex A1-450 chromato-integrator.

Chromatographic Procedure

150 mM sodium hydroxide was used as the eluant, made up with distilled, deionised water and vacuum filtered. The flow rate was 1 ml/min at a pump pressure between 1500 and 1600 psi. Run time was 20 minutes.

Sample Preparation

Samples were filtered with a Titan 2 µm pore nylon membrane HPLC syringe filter (Scientific Resources Inc., New Jersey, USA) into 2 ml screw top vials.

3.4.4 Sensory

Aroma, taste and appearance of end product cider is of extreme importance when studying the parameters of cider maturation. All the analysis is of little consequence, if the product is not found to be acceptable by the consumer.

Each sample was chilled slightly to 18-20°C prior to tasting. Approximately 20 ml of each sample was decanted into 125 ml wine flutes and put before the members of the tasting panel.

The panel consisted of up to ten regular semi -trained tasters, who recorded their observations with the help of the descriptives as set out in Appendix III. Each reporting of these features was then collated under the general headings `Acidic', `Aromatic', `Astringent', `Body', `Fatty', `Oxidised', `Solventy' and `Sulphury' and a rating on the scale 0 to 10 was allocated to each category for each sample. From these results, cider flavours were crudely assessed and compared.

CHAPTER FOUR

MICROBIOLOGICAL AND BIOCHEMICAL CHANGES IN COMMERCIAL CIDER DURING STORAGE

4.1 INTRODUCTION

Mature cider flavour is dependent upon complex interactions between physical, chemical and microbiological components of the cider during post fermentation storage. Conditions under which harsh flavour notes of newly fermented cider are reduced and a more mellow, mature product is formed, remains an area for empirical judgement. These process dynamics are little understood when compared to alcoholic fermentation, yet in many ways, the primary fermentation serves to provide a product base, with character developing during maturation. That is, final quality can be affected (positively or negatively), as much by storage as the style of the initial medium and fermentation.

Depending on storage conditions, changes in equilibrium between esters, acids, alcohols and other components of cider occur even during a short period of storage (Williams, 1989). Temperature, oxygenation, sulphur dioxide, acid content, residual sugars and most importantly the microbial population of cider interact to bring about these changes (Fredette, 1970).

Cider possesses a complex microflora, consisting of culture and wild *Saccharomyces* species and non-*Saccharomyces* species, such as *Pichia, Brettanomyces, Candida, Debaryomyces* and *Kloeckera* species (Beech, 1972b; Beech, 1993b). Bacteria present, include acetic acid bacteria, especially *Acetobacter xylinum* and lactic acid bacteria. Homofermentative *Lactobacillus plantarum* are commonly found in cider (Beech and Carr, 1977), as well as heterofermentative rods of *Lact. brevis, Lact. pastorianus* and *Lact. collinoides* (Beech and Carr, 1977). Heterofermentative cocci of the genus *Leuconostoc* and homofermentative cocci of the genus *Pediococcus* may also be

present. The sources of this microflora are predominantly associated with the cider fruit and its harvesting, along with those acquired from milling and pressing equipment (Beech and Carr, 1977; Beech, 1993a).

Micro-organisms present in storage cider may have beneficial or negative effects on end product aroma and flavour. Acetic acid bacteria are considered spoilage organisms and are, therefore, undesirable (Joyeux *et al*, 1984: Drysdale and Fleet, 1989). They increase volatile acidity to unacceptable levels and generate an unpleasant, vinegary aroma and flavour. Yeast sedimentation and autolysis results in the release of free amino acids, ammoniacal nitrogen, ribose, phosphate and Group B vitamins into the cider and is also associated with the appearance of unpleasant odours (Chatonnet *et al.*, 1991). This is generally considered undesirable in cider, although some winemakers believe that storage on the lees and hence autolysis, enhances the flavour complexity, without deacidification by malo-lactic fermentation (Dubourieu, 1986).

Other agents of cider disorders are considered to be *Pseudomonadaceae*, *Acetobacter*, *Hansenula anomala* and some species of *Lactobacillus* (Fredette, 1970), while strains of *Pediococcus cerevisiae* and *Leuconostoc mesenteroides* can form slimes which adversely alter the mouth-feel of the product (Beech and Carr, 1977). Generally, however, lactic acid bacteria are credited with positive flavour changes induced by malo-lactic fermentation which contributes to deacidification and improvement of organoleptic quality by increasing the cider's flavour complexity (Davis *et al.*, 1985; Kunkee, 1967). The degree of interaction between these micro-organisms will be dependent upon intake and excretion of nutrients (Etievant *et al.*, 1983), with a notable interaction between yeast and lactic acid bacteria widely reported (Carr, 1959; Beech, 1972b; Passmore and Haggett, 1973; Beech and Carr, 1977; Davis *et al.*, 1985; Ribereau-Gayon, 1985; Salih *et al.*, 1988; Salih *et al.*, 1990; Makanjuola *et al.*, 1992; Henick-Kling and Yun Hee Park, 1993).

This research investigates microbial profiles in maturing cider, after malo-lactic fermentation has been completed. The equivalent stage in wine making is sometimes referred to as conservation. Metabolism of chemical constituents after malo-lactic fermentation in cider is even less studied than in wines. Whilst types of micro-organisms

that may be found in ciders are well documented (Beech, 1972b; Beech and Carr, 1977), there is little information on how these populations develop over an extended storage period. Such studies have however, been carried out to an extent for wine (Davis *et al.*, 1986; Salih *et al.*, 1990). Examination of yeast, acetic acid and lactic acid bacteria populations during an extended storage period, as well as organic acid and sugar profiles were surveyed in order to obtain an outline of events occurring during cider maturation after malo-lactic fermentation.

4.2 EXPERIMENTAL

4.2.1 Maturing Cider under Investigation - May to July

The cider studied was fermented from fresh apple juice. The finished cider had a specific gravity of 0.996, ethanol at 9.7% v/v and less than 10 mg/l free SO_2. The cider was racked off its lees after attenuation and transferred into a cleaned 10 000 gallon (45400 l) oak wood storage vat, from which weekly samples were collected over 10 weeks from the beginning of May to early July.

Samples for microbial analysis were collected 10 cm below the liquid surface and also via a sterilisable sampling point, 1 m above the vat base (the height between the two ports was 6 metres). At the same time, a sample for chemical analysis was collected from this latter point.

4.2.2 Maturing Cider under Investigation - August to October

A second 10,000 gallon volume of storage strength General Blend cider, fermented from diluted apple juice concentrate, was racked off its lees into the same oak wood storage vat used in the previous study. This cider had similar specification to the previous cider base. Again samples were collected on a weekly basis from the top and bottom of the vat from the middle of August to the middle of October. Samples for microbial, physical and chemical analysis were collected from both sampling points.

4.2.3 Sample Analyses

Microbiological analysis was performed in accordance to the methods detailed in Section 3.1.1 and Appendices I and II. The methods for organic acid and sugars analyses are

detailed in Section 3.4.2 and Section 3.4.3 respectively. The method for flavour compound analysis is detailed in Section 3.4.1.

4.3 RESULTS

4.3.1 Maturing Cider - May to July

The frequency of occurrence of various species of yeast and lactic acid bacteria at separate stages of vinification has been reported (Fleet *et al.*, 1984), while the development of micro-organisms post alcoholic and malo-lactic fermentations is not well documented in either wine or cider.

4.3.1.1 Microbiological Profiles

Figure 4.1 shows the total number of micro-organisms in each of the four microbial groups enumerated over a ten week storage period, from the beginning of May to early July.

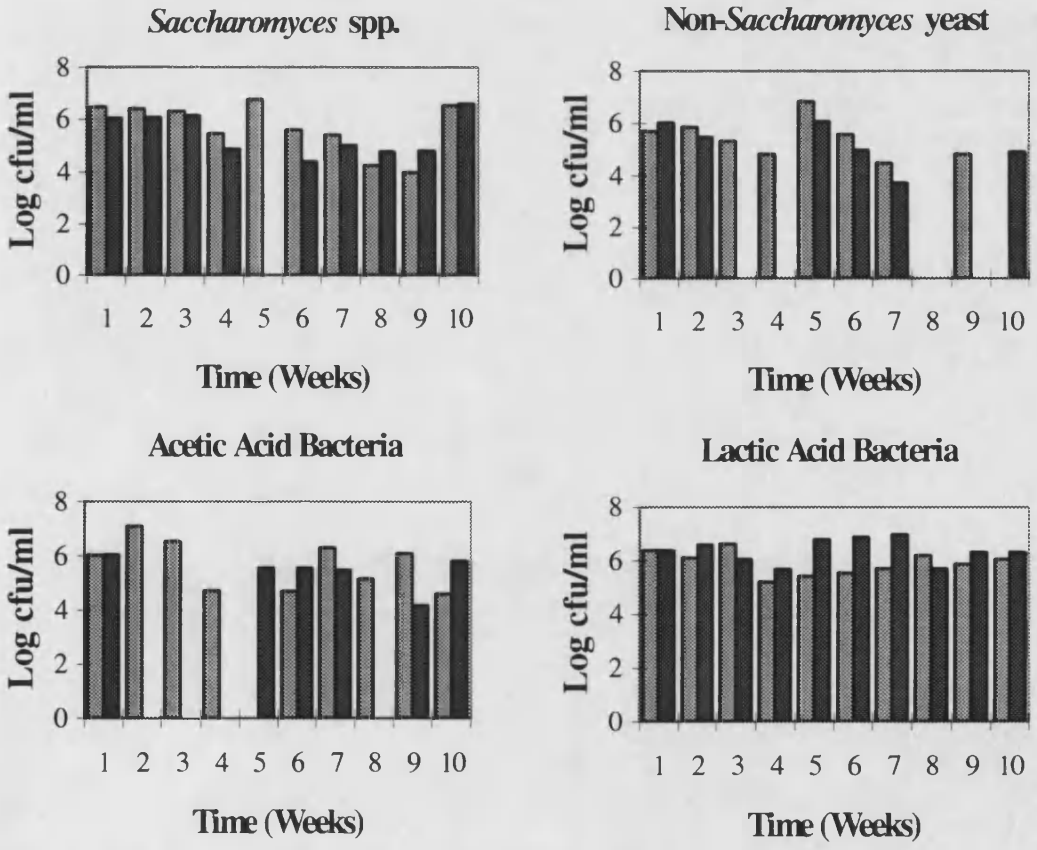

Figure 4.1 Top (▒) and base (■) microbial profiles of *Saccharomyces*, non-*Saccharomyces* yeast, acetic acid bacteria and lactic acid bacteria during ten weeks of storage of strong commercially fermented cider in a wood vat.

Using the paired t-test (Miller and Miller, 1988), there was no significant difference in the levels of acetic acid bacteria or either of the yeast populations at top and base of the vat ($P>0.05$). There was however a significant difference between lactic acid bacteria at the top and base of the vat ($P<0.05$).

An even distribution of *Saccharomyces* yeast in the cider throughout the storage period was observed, with a gradual decline in numbers as maturation progressed.

There were consistently more non-*Saccharomyces* yeast and acetic acid bacteria detected in cider from the top of the vat compared with cider from the base. A gradual decline in the non-*Saccharomyces* population throughout maturation was also detected.

The population of lactic acid bacteria remained constantly high throughout the maturation period. A significantly greater population of these bacteria was detected in samples collected from the base of the vat than at the top.

4.3.1.2 Organic Acid Profile

Malic acid can be degraded during fermentation by yeast to acetic and fumaric acids, while citric acid may be degraded to acetic acid during malo-lactic fermentation. The metabolism of components in cider after malo-lactic fermentation is largely unstudied, although some work has been undertaken in wine (Davis *et al.*, 1986).

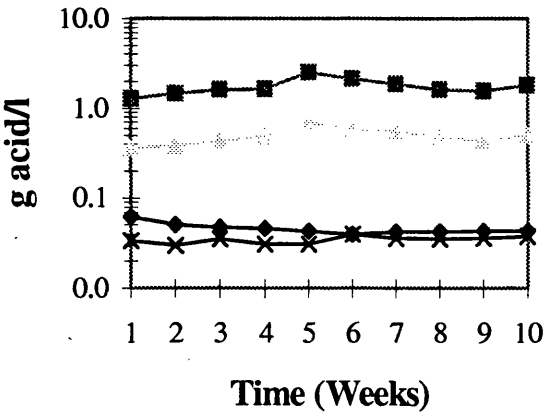

Figure 4.2 Malic (◆), lactic (■), acetic (△) and citric (X) acid profiles in maturing cider collected one metre from the base of the maturation vat.

A very gradual decline in concentration of malic acid throughout maturation was observed, while the concentration of citric acid remained constant. A trend to increase acetic acid and lactic acid concentrations was observed over the ten week period, as shown in Figure 4.2. This may be linked with an increase in the population of lactic acid bacteria at that time or a decline in *Saccharomyces* yeast.

4.3.1.3 Sugar Profile

Utilisation of hexose and pentose sugars by lactic acid bacteria during malo-lactic fermentation was reported by Davis *et al.*, (1986).

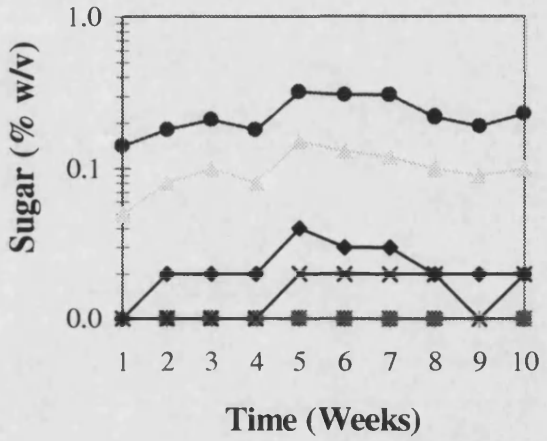

Figure 4.3 Glucose (◆), fructose (■), sucrose (△), maltose (●) and *iso*-maltose (**X**) concentrations determined in maturing cider over a ten week period.

Figure 4.3 shows that no fructose was detected in the cider throughout this study. *Iso*-maltose remained undetected until week five, after which a small, residual concentration was detected. There was a gradual increase in maltose, sucrose and glucose, peaking at week five, then declining in concentration gradually during the remaining maturation period.

4.3.1.4 Flavour Modifications

Only six of the compounds that were selected for identification by the GC flavour analysis method were found in this investigation of cider fermented from fresh apple juice. Figure 4.4 illustrates changes detected in the concentration of five of these compounds throughout

maturation. In addition, 4-ethyl phenol was present throughout maturation at a concentration of 2 mg/l.

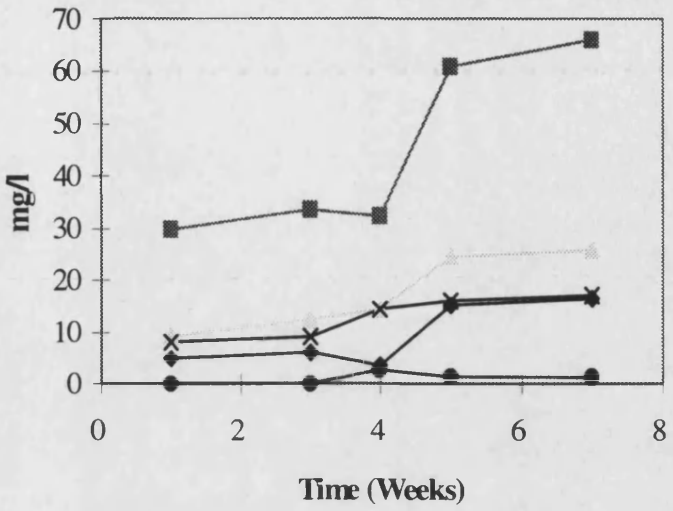

Figure 4.4 Changes in concentration of 2-methyl-1-butanol (■), ethyl lactate (△), 2-phenyl ethanol (X), hexanal (◆) and 4-ethyl catechol (●) found in maturing cider over a seven week period.

Initially detected at week four, 4-ethyl catechol then remained present in the cider. 2-phenyl ethanol showed a gradual increase in concentration, perhaps increasing more rapidly between weeks three and four. 2-methyl-1-butanol, ethyl lactate and hexanal all showed a dramatic increase in concentration (~ 48%, 44% and 67% respectively) between week four and five, and continued to be detected at those concentrations for the rest of the investigation.

4.3.2 Maturing Cider - August to October

The previous study had been performed on cider fermented from fresh apple juice, during the warmer months of the year. An additional maturation study of cider fermented from diluted apple juice concentrate, was undertaken. Microbiological profiles at the top and base of the vat were determined for these samples, as were organic acid and sugar profiles. In addition, pH, temperature and dissolved oxygen were assessed for these cider samples. Flavour analysis by gas chromatography was performed on samples collected from the base of the vat.

4.3.2.1 Microbiological Profiles

Figure 4.5 illustrates the total number of micro-organisms in each of the four microbial groups enumerated over an eleven week storage period, from the middle of August to the middle of October.

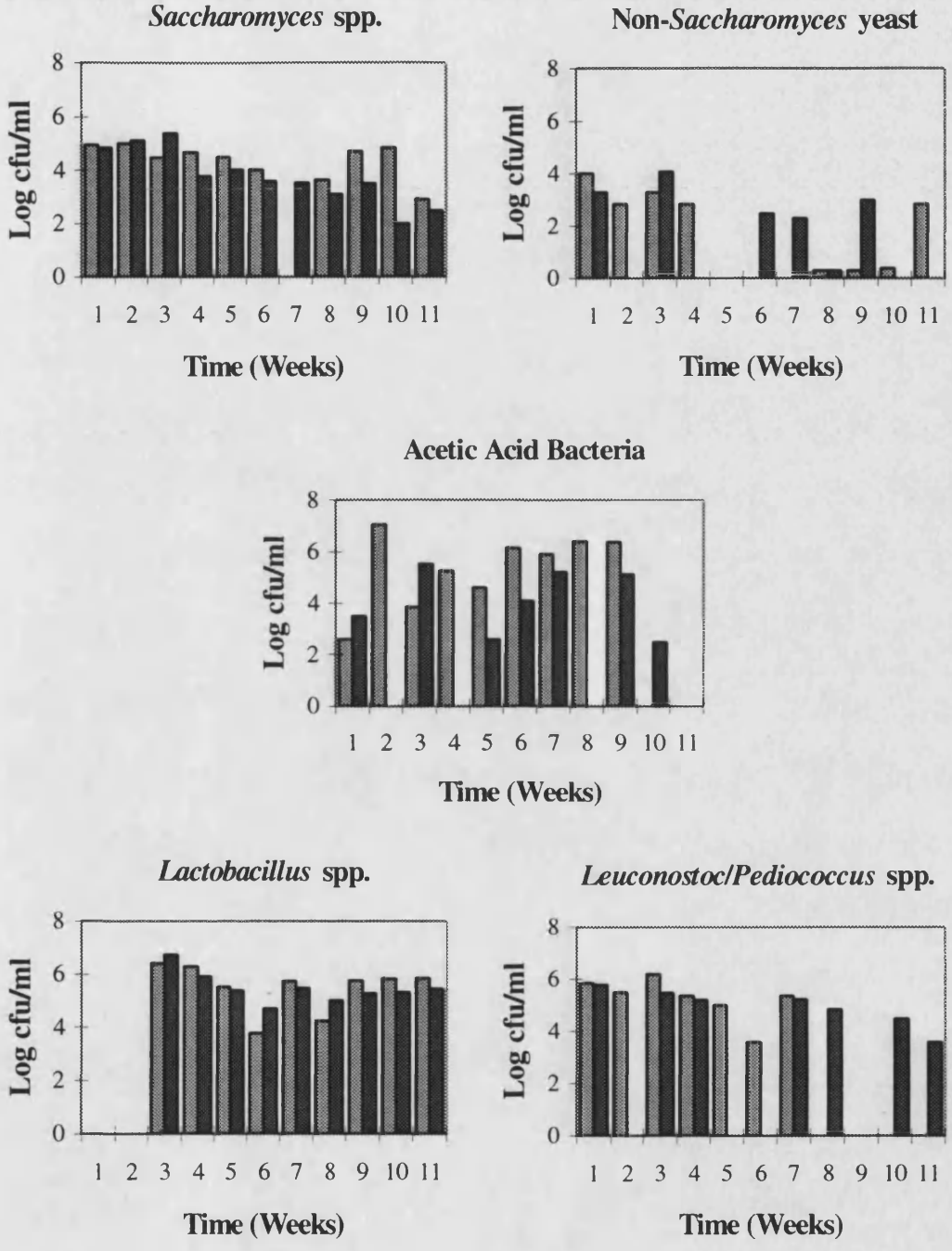

Figure 4.5 Total microbial numbers in maturing cider, top (▒) and base (■) of wood vat.

Using the paired t-test (Miller and Miller, 1988), there was no significant difference in either of the lactic acid bacteria populations or either of the yeast populations at top and base of

the vat ($P>0.05$). The was however a significant difference between acetic acid bacteria at the top and base of the vat ($P<0.05$).

There were lower levels of micro-organisms throughout this second maturation, than detected in cider fermented from fresh apple juice. In addition, there was generally a higher number of yeast enumerated from cider collected from the top of the vat than in cider from the base.

For the first four weeks, non-*Saccharomyces* yeast were readily isolated from the top of the vat, but then remained undetected until week eight. A film of non-*Saccharomyces* yeast and acetic acid bacteria was observed to develop over the surface of the cider. By week five, this film was approximately 2 cm thick, after which it gradually formed clumps and then redispersed. Non-*Saccharomyces* yeast were not, however, detected in substantial concentrations in samples collected from the top sampling port.

Acetic acid bacteria were detected consistently at high levels from cider collected from the upper sampling port for almost the length of maturation, with numbers only declining during the last two weeks, where an increase in non-*Saccharomyces* yeast was also noted. In cider collected from the lower sampling port, acetic acid bacteria varied markedly in numbers and were generally less than in the cider from the top of the vat. Overall numbers of acetic acid bacteria from the bottom of the vat were significantly ($P<0.05$) lower than in the top vat samples.

Possibly the most important observation made concerned the populations of lactic acid bacteria. In this second investigation, *Lactobacillus* spp. and *Leuconostoc/Pediococcus* spp. were differentiated by cell morphology (Appendix II), thus enabling separate profiles of rod and coccal forms to be determined. As can be seen from Figure 4.5, no *Lactobacillus* spp. were detected for the first two weeks of maturation, but they rapidly achieved a cell density of approximately 10^6 cfu/ml by the third week and remained at this elevated level throughout the cider for the duration of maturation.

Coccal forms of cider lactic acid bacteria were, however, detected at a cell density of 10^6 cfu/ml at the outset of maturation, apparently homogeneously distributed throughout the

cider. The population declined to undetectable levels in upper cider samples over the first seven weeks of maturation. A more complex pattern was elucidated from samples collected from the lower sampling port. The population disappeared to undetectable levels in the second week, reappearing at almost the same population density as before, one week later. This re-emerged population declined rapidly to undetectable levels again. This cycle was repeated twice more before the end of sampling.

4.3.2.2 Organic Acid Profiles

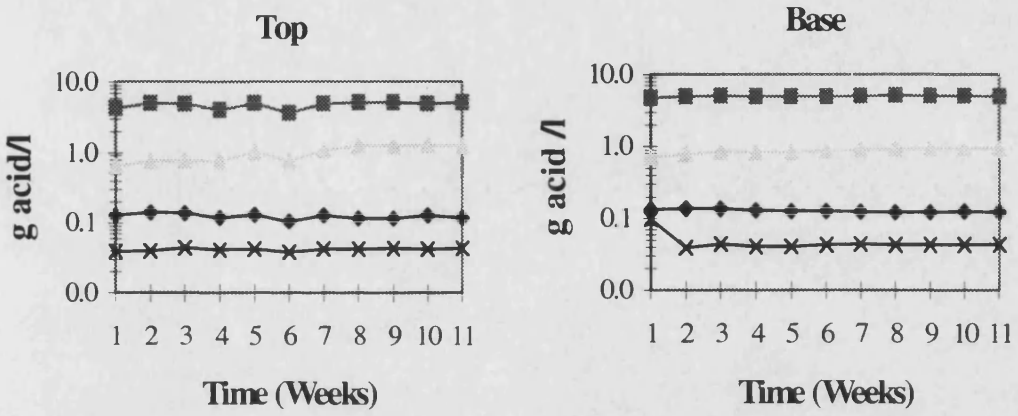

Figure 4.6 Malic (◆), lactic (■), acetic (△) and citric (**X**) acid profiles in maturing cider collected one metre from the base of the maturation vat.

Figure 4.6 illustrates the stability of all the organic acids analysed for (malic, lactic, acetic and citric acids) in cider during this maturation. It is important to note that the concentration of lactic acid in the cider of this investigation was double that detected in the cider in the first study.

4.3.2.2 Sugar Profiles

Figure 4.7 shows that fructose and *iso* maltose were not detected in this cider throughout maturation. Sucrose and maltose were detected initially, but disappeared within a week of maturation. Glucose, however, was detected throughout the cider for the duration of maturation. This profile is very different from that determined in the first study in which glucose was not detected and maltose and sucrose occurred throughout maturation.

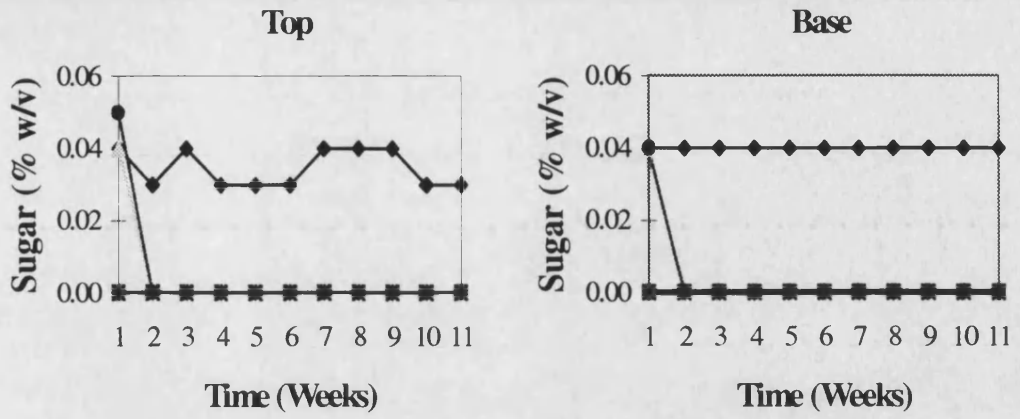

Figure 4.7 Glucose (◆), fructose (■), sucrose (△), maltose (●) and *iso* maltose (**X**) concentrations determined in maturing cider over an eleven week period.

4.3.2.3 Flavour Modifications

Octanoic acid, *iso*-amyl acetate, 4-ethyl guaiacol and undecanal were all detected in this cider at constant concentrations of 5, 1, 1 and 4 mg/l respectively, for the duration of the maturation. Other compounds detected in this cider are shown in Figure 4.8.

Ethyl lactate and 2-methyl-1-butanol were again the two flavour compounds that changed most dramatically during storage, increasing after the sixth week of maturation. The latter compound increased in two stages, initially by 68% between week one and two, then by about 40% between weeks six to nine. Hexyl acetate increased gradually during storage, as did ethyl octanoate. In this cider, 2-phenyl ethanol varied little throughout maturation.

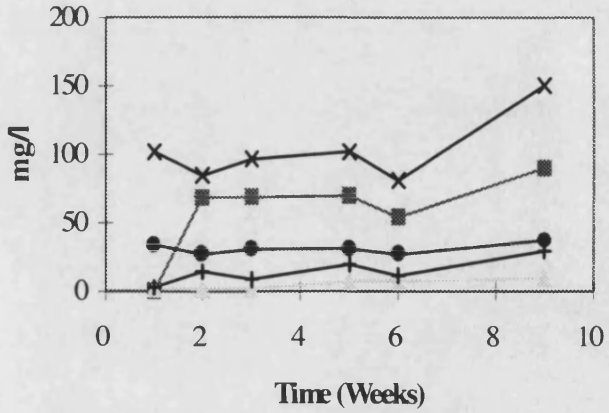

Figure 4.8 Changes in concentration of ethyl lactate (**X**), 2-methyl-1-butanol (■), ethyl octanoate (+), hexyl acetate (△) and 2-phenyl ethanol (●) found in maturing cider over a nine week period.

4.3.2.4 Physical Profiles for Summer-Autumn Maturation

Temperature

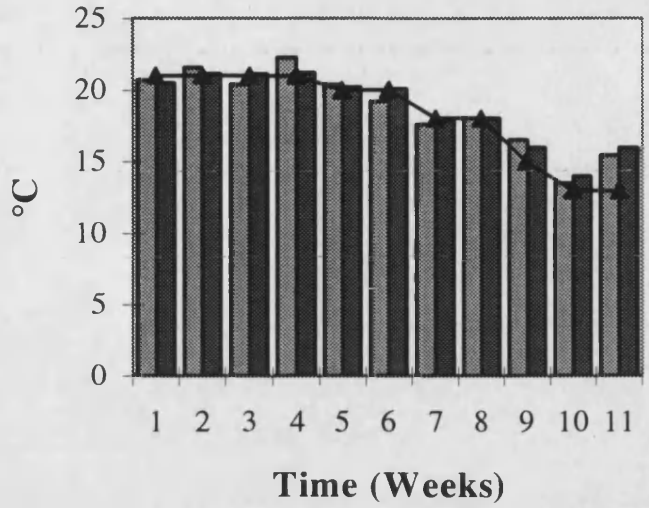

Figure 4.9 Temperature profiles of cider at top (▒) and base (■) of maturation vat and ambient temperature of vat house (▲).

There was no significant difference between top and bottom temperatures over the storage period, using the paired t-test (Miller and Miller, 1988) $P>0.05$, although a 7° to 8 °C drop in both ambient and cider temperatures was recorded during maturation (Figure 4.9).

Wooden storage vats are held in an enclosed, but unheated vat house. As biochemical and microbial activities are temperature dependent, maturation profiles will be influenced therefore by residual heat from the exothermic fermentation reaction and environmental temperature.

pH

Factors affecting pH during maturation are physico-chemical and microbial, with both growth activity and death of individual populations of the microflora generating changes in pH, as well as changes in equilibrium between acids, alcohols, esters and other cider components (Williams, 1989).

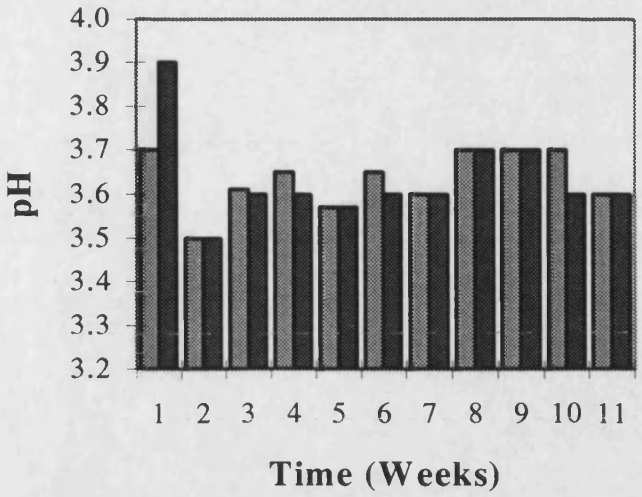

Figure 4.10 pH profiles of cider at top (▨) and base (■) of maturation vat.

There was no significant difference between top and bottom pH values over the storage period, using the paired t-test (Miller and Miller, 1988) $P>0.05$. A gradual decrease in acidity as maturation progressed can be observed from Figure 4.10.

Dissolved Oxygen

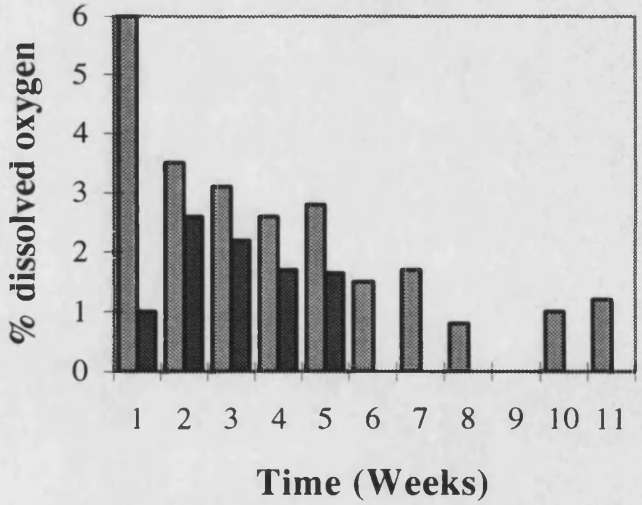

Figure 4.11 Dissolved oxygen profiles of cider at top (▨) and base (■) of maturation vat.

There was a significant difference ($P<0.05$) between the dissolved oxygen content at the top and bottom of the vat over the storage period, using the paired t-test (Miller and Miller, 1988).

Cessation of fermentation and racking the cider from the lees by pumping it to the storage vat, would have created the initial slightly aerobic conditions seen in Figure 4.11. As storage progressed, the levels of dissolved oxygen in the cider diminished, decreasing suddenly between weeks five and six to a relatively stable level of less than 2% dissolved oxygen at the top and 0% at the base.

4.4 DISCUSSION

With the aid of selective culture media, it was possible to observe that total numbers of micro-organisms were reasonably constant during maturation, but there were significant variations in types of micro-organisms. Evolution of these populations is a consequence of several factors including the micro-climate, hygiene, milling, pressing and apple variety used at the cider factory. All these factors contribute to the development of a complex microflora.

Fewer micro-organisms were isolated from cider fermented from rediluted apple juice concentrate than fresh apple juice, with the exception of lactic acid bacteria. The populations of these micro-organisms were isolated at a concentration of approximately 10^6 cfu/ml from both maturing ciders. It is most probable that the overall higher population densities detected in fresh apple juice-derived cider were a feature of the apple juice. Firstly, microbial populations in this medium would be higher than those of rediluted concentrate, as destruction of micro-organisms would have occurred during the concentration process. Secondly, the apple juice contains higher levels of oxygen entrapped by pulp particles (Pollard *et al.*, 1967; Klingshirn *et al.*, 1987), and these aerobic conditions would encourage growth of acetic acid bacteria and oxidative yeast.

There was an even distribution of *Saccharomyces* yeast throughout maturation in both ciders studied, although these populations gradually declined as storage progressed. These yeast are dominant at the end of fermentation and during storage as a consequence of their ethanol tolerance, as also reported for French cider (Salih *et al.*, 1988). The decline in yeast populations between the beginning and end of maturation may be considered due to a lack of nitrogen and vitamins, as well as competition for nutrients with other yeast and bacteria present (Carr, 1959; Beech and Carr, 1977;

Salih *et al.*, 1988). Ethanol and low molecular weight substances are also implicated in the role of yeast inhibition and death, particularly short chain fatty acids (Ribereau-Gayon, 1985).

Although *Saccharomyces* spp., from both the natural microflora and the strain used for the alcoholic fermentation, are moderately flocculating and will gradually sediment out of suspension, there was no evidence of an increase in *Saccharomyces* population at the base of the vat as maturation progressed. Some *Lactobacillus* spp. have been thought to enhance the ability of yeast to flocculate (Momose *et al.*, 1969; Beech, 1972b), although any aggregation may suppress sugar utilisation by inhibiting diffusion of nutrients into cells, and result in cell death (Makanjuola *et al.*, 1992). Furthermore, when lactic acid bacteria are in intimate contact with yeasts, autolysis may be accelerated by acid production (Passmore and Haggett, 1973). Yeast have been shown to associate with both *Leuconostoc* spp. and *Lactobacillus* spp. (Beech, 1972b; Passmore and Haggett, 1973).

A gradual decline in non-*Saccharomyces* yeast was recorded in the fresh juice-derived cider, while a more dramatic decrease was observed in the concentrate-derived cider after the fourth week. Competition for nutrients and dissolved oxygen, especially with acetic acid bacteria, was apparent, as the population decline corresponded to surface film development and a decrease in dissolved oxygen content of cider.

There were more non-*Saccharomyces* yeast nearer the surface of the cider, than at its base, as these micro-organisms tend to possess a greater requirement for oxygen than *Saccharomyces* species, that is, they have oxidative or weakly fermentative metabolisms. Certain non-*Saccharomyces* yeast, in particular *Pichia membranefaciens* and *Candida valida*, have been reported previously to develop when there is a ready supply of oxygen, particularly on the surface of stored cider and at sample ports (Beech and Carr, 1977; Rosini, 1984).

As maturation proceeds, yeast populations can excrete nitrogen compounds, including aromatic amino acids, peptides and nucleotides into the cider (Beech, 1972b; Beech and Carr, 1977), and as they autolysed, their cell contents would also be released.

Utilisation of these nutrients by the residual population of non-*Saccharomyces* yeasts, may have led to the increasing population of non-*Saccharomyces* spp. at the base. Their reappearance may also be linked with the decline in acetic acid bacteria.

In addition, some bacteria secrete anti-yeast compounds (Beech, 1972b). One such well known inhibitor is acetic acid which affects growth of yeasts, including *Kloeckera* and *Candida* species (Drysdale and Fleet, 1989). Ethanol is metabolised to acetic acid by acetic acid bacteria, which can accelerate yeast death as this acid decreases the intracellular pH of yeast more rapidly than any other fatty acid (Kaneko and Yamamoto, 1966). *Acetobacter rancens* and *A. aceti* are known to inhibit yeast in this manner (Beech and Carr, 1977) and an *Acetobacter* resembling *A. rancens* has been reported to kill yeasts anaerobically, causing autolysis (especially in the presence of wood) with a short lived, low molecular weight toxin (Gilliland and Lacey, 1966). This toxin was found to be lethal to a number of species of *Saccharomyces*, *Hansenula anomala*, *Pichia fermentans*, *Shizosaccharomyces pombe*, *Zygosaccharomyces acidifaciens*, *Torula utilis* and *Candida mycoderma*.

As with non-*Saccharomyces* yeast, acetic acid bacteria were more prevalent at the top of cider. This again can be attributed to the presence of more oxygen at the top than at the base of the vat. The dissolved oxygen content of the cider rapidly decreased as the surface flora attained sufficient growth and activity to exclude oxygen from below the surface. Subsequently, the population of acetic acid bacteria during maturation will be competing for oxygen with the non-*Saccharomyces* yeast in the surface flora. The resurgence of the base populations of non-*Saccharomyces* spp. and acetic acid bacteria may be due to an increase in oxygen, most probably at the inspection/sampling port (Beech and Carr, 1977). Although not investigated in this study, a succession of acetic acid bacteria throughout cider and wine making, with *Acetobacter aceti* constantly present has been reported (Joyeux *et al.*, 1984; Salih and Suarez Diaz, 1990).

Lactic acid bacteria are considered amongst the most important micro-organisms involved in flavour changes during maturation of cider and wine, exerting an influence on both product quality (Crapisi *et al.*, 1987b) and bacterial stability (Crapisi *et al.*, 1987b; Davis *et al.*, 1988; Wibowo *et al.*, 1988). Under standard conditions, lactic

acid bacteria remain viable in wine during storage, showing no tendency for further growth and a slow, progressive decline (Lafon-Lafourcade *et al.*, 1983).

In fresh apple juice-derived cider, enumeration of the total lactic acid bacteria population illustrated their universal presence throughout storage cider. A gradual decline in this population was observed at the surface of the product. These microaerophilic and facultatively anaerobic micro-organisms could be dying because of excess oxygen levels at the surface, or from competition for nutrients with other micro-organisms, or through sedimention, either alone or attached to sedimenting yeast.

In the second investigation, bacillus and coccal forms of lactic acid bacteria were differentiated, thus producing a more detailed microbial profile. In wines, *Leuconostoc oenos* is generally the only species of lactic acid bacteria detected after alcoholic fermentation (Lafon-Lafourcade *et al.*, 1983; Davis *et al.*, 1988).

The population of *Lactobacillus* had a latent period after fermentation, with surviving bacteria present in undetectable numbers. This two week period was followed by substantial proliferation, as reported for wine by Davis *et al.*, (1986). Beech and Carr (1977) reported that towards the end of fermentation, *Lactobacillus plantarum* and *Lact. mali* are prevalent, gradually replaced by *Lact. collinoides*. In this current work, this population remained at a cell density slightly below 10^6 cfu/ml throughout maturation. Davis *et al.*, (1986) reported that after malo-lactic fermentation, lactic acid bacteria remained in a viable, non-proliferating state for long periods.

Lactic cocci, initially present at the top of the vat in high numbers, gradually decreased. This was probably due to a combination of reported sensitivity to sulphur dioxide (Beech, 1993a) and lack of essential vitamins (Carr, 1958). Lactic acid bacteria are highly sensitive to sulphur dioxide, which is produced in significant amounts by some yeasts (Davis *et al.*, 1985; Henick-Kling and Yun Hee Park, 1993), with cocci more sensitive to than rods (Beech, 1972b). They also universally require pantothenate, nicotinic acid, riboflavin and thiamine, which are released into the cider during yeast autolysis (Carr, 1958).

Cycles of growth and death were shown to occur in the *Leuconostoc/Pediococcus* population. After a decline in numbers, an exponential growth phase was observed, then a second phase of decline was noted, with no stationary phase. A similar cryptic growth was reported in beer by Fernandez and Simpson (1994). They determined that the viable cell count fell by 99.9% during the decline phases and suggested that as the cells died, the released nutrients enabled new cells to grow. Cryptical cells were reported to possess short lag phases.

In the first study, increases in acetic and lactic acids, was observed at the fifth week. It is possible that these increases were influenced by an increase in the lactic acid bacteria population observed at that time through their utilisation of glucose or fructose (Davis *et al.,* 1988). There was, however, no corresponding decrease in these mono-saccharides at this time. Indeed, a slight increase in glucose was recorded. These changes were not observed in concentrate-derived cider, in which the organic acid profile remained constant throughout maturation. In addition, the gradual decrease in acidity shown by the pH profile of the concentrate-derived cider, was not, therefore, linked with any of the organic acids detected. Esterification or other changes in chemical composition of the cider may influence this pH profile.

No fructose or *iso*-maltose were detected in either cider studied. In the fresh juice-derived cider, a small, but insignificant increase in sucrose was observed at week five, the level of which decreased gradually thereafter. Indeed, maltose was the only sugar detected in any quantity. No reason can be found for the three week increase of this sugar during mid maturation. In the concentrate-derived cider, glucose was the only sugar detected after the first week of maturation, with no changes throughout storage.

In wines, it has been found that concentrations of glucose and fructose may increase during malo-lactic fermentation. These sugars may be derived from residual enzymatic activities of grapes and yeast, hydrolysing sucrose, trehalose or phenolic glycosides (Davis *et al.,* 1986; Davis *et al.,* 1988). Other sugars, in particular ribose, arabinose and *myo*-inositol are of interest in wines.

Flavour compounds associated with fermentation and yeast metabolism have been the subject of many investigations over the years, not only in cider (Williams and Tucknott, 1971; Williams and Tucknott, 1977; Leguerinel *et al.*, 1988) but also wine (Daudt and Ough, 1973; Bertuccioli and Viani, 1976; Baumes *et al.*, 1986; Nykanen, 1986; Herraiz *et al.*, 1991; Chatonnet *et al.*, 1993) beer and distilled beverages (Suomalainen and Lehtonen, 1979).

Changes in flavour during maturation, however, are still undefined to a great extent. Beverages contain a large number of minor compounds which appear after fermentation, in particular, aldehydes, acids, esters, furfural and some coloured compounds (Nykanen, 1986). In wines, it was found that the concentration of several esters, such as butyl acetate, ethyl lactate, ethyl octanoate, 3-methylbutyl lactate, methyl decanoate and phenylethyl hexanoate increased after malo-lactic fermentation (Durr, 1986).

Owing to delays in GC analysis and subsequent sample deterioration, identification of flavour compounds was limited, but nevertheless, those compounds identified provided interesting results. In these studies, fewer flavour compounds were identified in fresh juice-derived cider than concentrate-derived cider. This may be a factor of the sample preparation techniques or that many of the compounds present were not identified by the chromatography method.

In both maturation studies, ethyl lactate and 2-methyl-1-butanol increased. Durr (1986) reported an increase in ethyl lactate after malo-lactic fermentation in wines. An increase in the former compound is attributed to chemical equilibration during ageing (Williams, 1989) by the esterification of lactic acid (Etievant and Williams, 1984). Etievant and Williams (1984) also reported an increase in 2- and 3-methyl-1-butanol during the ageing of Beaujolais wines.

2-Phenyl ethanol remained at a static level throughout maturation of concentrate-derived cider and increased slightly during the maturation of the fresh juice-derived cider. 2-Phenyl ethanol and its esters are considered to contribute to the base aroma of cider (Williams and Tucknott, 1971; Williams, 1974; Durr, 1986).

In the fresh juice-derived cider maturation, hexanal, an aldehyde associated with green, grassy aromas, was found to increase, while 4-ethyl phenol, associated with a heavy, cresol-like aroma remained constant. This latter compound is thought to arise from bacterial action on coumarin, a phenolic compound, although this could not be confirmed by this investigation (Williams and Tucknott, 1971).

In concentrate-derived cider, octanoic acid, a C-8 fatty acid and *iso*-amyl acetate, both synthesised by yeast during alcoholic fermentation, remained at constant levels throughout maturation. Undecanal and 4-ethyl guaiacol, a phenolic compound that imparts a heavy, phenolic aroma to the cider, were found to remain static during maturation. Ethyl catechol, identified in the fresh juice-derived cider, is possibly the precursor to ethyl guaiacol (Williams and Tucknott, 1971) although no comment regarding this information can be made with reference to this investigation.

Hexyl acetate, an ester associated with fruity, apple aroma, remained at a constant level throughout this maturation. In Beaujolais wines (Etievant and Williams, 1984) and Fino sherries (Williams, 1989), this compound decreased during ageing. Ethyl octanoate, which increased gradually throughout the maturation, was reported to increase during ageing of sherries (Williams, 1989).

Maturation flavour is extremely complex, where changes induced initially may be reversed later and are a consequence of yeast, bacteria and/or chemical activities. More sensitive and extensive analyses of sugars, organic acids and, most importantly, volatile flavour compounds are required in order to attempt to understand some of these complex, subtle changes that are taking place during maturation of commercial cider.

4.5 SUMMARY

- Every cider maturation is unique, dependent upon many factors including the apple juice base used for fermentation, compounds synthesised during fermentation and microflora present in the cider.

- Maturing cider possesses a complex microflora, consisting of *Saccharomyces* and non-*Saccharomyces* yeast, acetic acid bacteria and lactic acid bacteria.

- Microbial populations vary throughout cider, surface cider containing more aerobic micro-organisms than those found in the bulk of the cider. In addition, these populations vary throughout cider maturation.

- Lactic acid bacteria, in particular *Leuconostoc/Pediococcus* spp. may have a significant role in cider maturation, as they have complex nutritional requirements and are capable of utilising residual carbohydrates, organic and phenolic acids, as well as compounds released by other micro-organisms.

- Ethyl lactate, 2-methyl-1-butanol and 2-phenyl ethanol are potential maturation marker compounds, easily detected in cider, the first two increasing dramatically as maturation progresses.

- It is recommended that volatile phenolic compounds, such as 4-ethyl phenol, 4-ethyl catechol and 4-ethyl guaiacol, their (bio)synthesis and influence on the aroma of mature cider, be the subject of further investigation.

CHAPTER FIVE

EFFECTS OF STORAGE PARAMETERS ON CIDER FLAVOUR

5.1 INTRODUCTION

Temperature, agitation, exposure to carbon dioxide or oxygen and the presence, or not, of particulate matter during maturation are examples of storage parameters that may have a direct or indirect influence on cider flavour. Prior to considering any process modifications, the effects of these non-microbiological parameters on cider flavour and microflora have to be assessed.

Cider is fermented from both fresh apple juice and diluted concentrate. Although both will contain apple pulp particles, fresh juice possesses a considerable quantity of pulp particles in suspension. Effects of particulate matter on higher alcohol formation during fermentation is well documented (Pollard *et al.*, 1965; Pollard *et al.*, 1966; Klingshirn *et al.*, 1987). The insoluble solids present in fruit based alcoholic beverages during fermentation influences the level of higher alcohols and esters. Beverages fermented from clarified juice often contain small amounts of higher alcohols and higher amounts of esters compared with those from an unclarified juice (Klingshirn *et al.*, 1987).

Effects of fermentation temperature on flavour development of a product is generally associated with microbial activities, as opposed to chemical changes *per se*, although volatile compounds with low boiling points may be lost at higher fermentation temperatures (Killian and Ough, 1979).

Exposure of cider to air during storage leads to undesirable changes in flavour and aroma. Auto-oxidation is a complex reaction cascade involving catalytic heavy metals and commences with the oxidation of phenolic compounds and leads to the development of acetals and aldehydes (Jones *et al.*, 1986). Acetaldehyde, acetic acid and ethyl acetate are produced from the oxidation of ethanol, increasing the volatile acidity of cider (Rapp and

Manery, 1986). All these oxidative changes are increased at higher temperatures, resulting in a loss of fruitiness and freshness (Rapp and Manery, 1986).

Fermented cider is frequently transferred between vessels during storage, either when racked off lees, fined, filtered or blended with other cider. This 'agitation' may affect dissolved oxygen and carbon dioxide content as well as increasing the homogeneous nature of the cider. The significance of these factors on product flavour has to be ascertained. Rodriguez *et al.*, (1990) reported that stirring the lees of Burgundy wines was practised as a means of achieving a flavour complexity similar to that produced by malo-lactic fermentation.

Effects of high, ambient and low temperatures, degrees of insoluble solid content, aeration (and anaerobiosis) and agitation on cider flavour during storage were explored.

5.2 EXPERIMENTAL

5.2.1 Effects of Different Storage Temperatures on Cider Flavour

The effects of three different storage temperatures on cider flavour were investigated; 5°, 15° and 25°C, corresponding to cold, ambient, high temperature storage, respectively.

5.2.1.1 Temperature #1

Recently racked cider from a commercial fresh juice fermentation was collected in sterile 5 gallon polyethylene casks, the lids of which had one way valves, which allowed evolved carbon dioxide to escape while preventing air penetration. Polycasks were stored at each temperature for six months.

5.2.1.2 Temperature #2

Cider fermented in the laboratory from concentrated apple juice was microfiltered directly into sterile maturation vessels (Section 3.2.5). Two maturation vessels were stored at each temperature for three months.

5.2.2 Effects of Filtration\Pulp Particle Size on Cider Flavour during Storage

5.2.2.1 Filtration #1

10 litres of fresh apple juice was fermented in the laboratory as detailed in Section 3.2.2. Cider fermented from the unfiltered fresh juice was divided into three quantities: One third of which was racked off the lees into a sterile maturation vessel; One third was aseptically filtered though a 0.45 µm Millipore membrane unit and the remaining cider was sterile filtered with a Romicon ceramic 500,000 M.W.. All were stored at 15°C for six months.

5.2.2.2 Filtration #2

Cider fermented in the laboratory from concentrated apple juice was divided into three equal volumes; a third was racked off the lees, a third was microfiltered and a third was ultrafiltered directly into sterile maturation vessels (Section 3.2.5). Two maturation vessels of each filtration were stored at 15°C for three months.

5.2.3 Effect of Aeration and Anaerobiosis on Cider Flavour during Storage

5.2.3.1 Aeration/Anaerobiosis #1

Cider from a commercial fresh juice fermentation was sterile filtered (Section 3.2.5) and divided equally between sterile maturation vessels. Filtered air was bubbled through cider in two of the maturation vessels for 10 minutes and fitted with an airlock (Section 3.3). Filtered carbon dioxide was bubbled through cider in the two remaining maturation vessels for 10 minutes and was fitted with an airlock (Section 3.3). All vessels were then stored at 15°C for four months.

5.2.3.2 Aeration/Anaerobiosis #2

Cider fermented in the laboratory from concentrated apple juice was sterile filtered (Section 3.2.5) and decanted into sterile maturation vessels. A headspace of approximately one quarter of the total volume remained in each vessel. Filtered carbon dioxide was continuously bubbled through the cider in one vessel at an approximate rate of 1 dm^3/min. Filtered air was bubbled continuously through cider in the second vessel at a similar rate and the third vessel was left to equilibrate with the atmosphere. The vessels were stored at 15°C for five weeks.

5.2.4 Effect of Agitation on Cider Flavour during Storage

Cider fermented in the laboratory from concentrated apple juice was sterile filtered (Section 3.2.5), decanted into sterile maturation vessels and blown over with filtered carbon dioxide. Cider in one maturation vessel was recirculated during storage via a peristaltic pump. Cider in the remaining vessel remained static. These vessels were stored at 15°C for three weeks.

5.2.5 Experimental Overview

In Figures 5.1 and 5.2, a summary of the experimental programs for cider derived from fresh juice and concentrate is presented. Ciders from different investigations, but stored under comparable conditions, are indicated by superscripts, e.g. a, b, c, d.

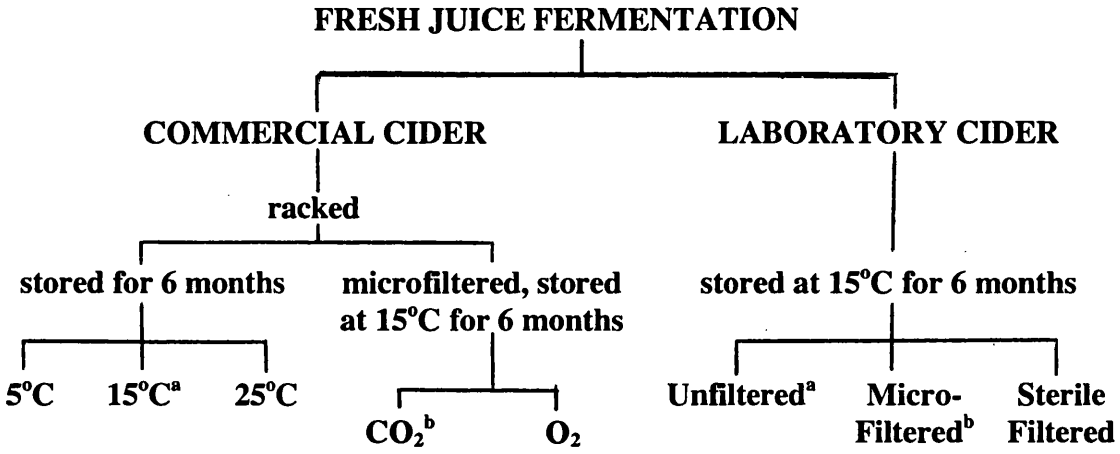

Figure 5.1: Summary diagram of investigations into effects of various storage parameters on flavour changes in fresh juice-derived cider.

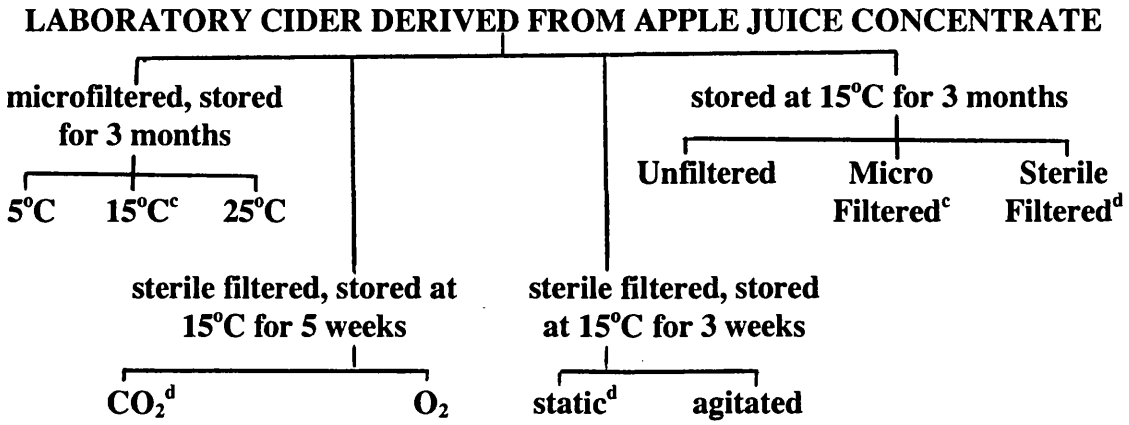

Figure 5.2: Summary diagram of investigations into effects of various storage parameters on flavour changes in concentrate-derived cider.

5.3 RESULTS

Statistical analysis of the levels of flavour compounds identified in cider samples was not performed owing to insufficient samples analysed and the large error in the sample preparation method (described in Section 3.4.1), which was determined to be approximately ± 20%. In order to assign a measure of importance of increase or decrease in flavour compound concentration during storage, an arbitrary figure of 20% was assigned.

Abbreviations: = no change; - ≥ 20% decrease; + ≥ 20% increase, - - >>20% decrease, + + >>20% increase (overall changes in concentration during storage).

5.3.1. Effects of Storage Temperature on Cider Flavour

5.2.1.1 Temperature #1

The commercial cider used in this investigation had undergone malo-lactic conversion prior to racking off the lees and storage.

Cider collected in sterile containers from a commercial cider fermentation, immediately after racking, showed a mixed population of micro-organisms (Table 5.1). This large, complex population may, itself, be influenced by storage temperature and may, in turn, instigate changes in flavour compounds.

Table 5.1: Micro-organisms present in commercial cider at outset of storage trial at three different temperatures.

MICROBIAL GROUP	LOG AVERAGE CFU/ML IN CIDER AT START OF STORAGE
Saccharomyces spp.	5.6
Non-*Saccharomyces* yeast	5.3
Lactic Acid Bacteria	5.5
Acetic Acid Bacteria	5.4

Table 5.2: Overall effects of storage temperature on flavour compounds in cider.

COMPOUND	5°C	15°C	25°C
hexanal	- -	- -	- -
octanal	=	=	+
nonanal	=	=	=
undecanal	=	=	=
heptanoic acid[4]	+	+	+
octanoic acid	=	=	=
2-methyl-1-butanol	=	=	=
hexanol	-	-	=
***iso*-amyl acetate**	=	=	=
hexyl acetate	=	=	=
ethyl-2-methyl butyrate	=	=	=
ethyl hexanoate	=	=	=
ethyl lactate[1]	- -	- -	=
ethyl octanoate	=	=	=
ethyl decanoate[2]	=	=	+
diethyl succinate	-	-	-
ethyl dodecanoate[3]	=	=	=
ethyl benzoate	+	+	-
phenylethyl acetate	-	- -	- -
2-phenyl ethanol	+ +	+ +	+ +
4-ethyl phenol	-	-	+

[1] see Fig. 5.3; [2] see Fig. 5.4; [3] see Fig. 5.5; [4] see Fig. 5.6;

Ten compounds were shown to remain constant throughout the six months storage period, and can therefore be considered not to be influenced by storage temperature (Table 5.2). Hexanal, diethyl succinate and phenylethyl acetate decreased during storage and 2-phenyl ethanol increased, but were apparently unaffected by temperature, as these changes occurred at all three temperatures.

Figures 5.3 to 5.6 illustrate changes in concentration of some compounds that appeared to be affected by storage temperature. Figure 5.3 illustrates changes in concentration of ethyl lactate during storage at the three temperatures. At the lowest and highest storage temperatures, an initial increase in ethyl lactate was followed by a gradual decline, whereas an initial decline proceeded a constant concentration, at 15°C.

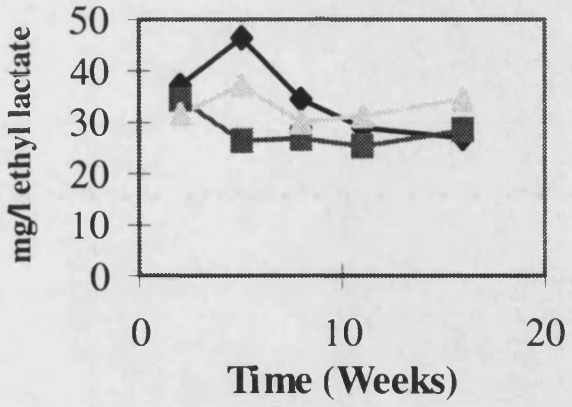

Figure 5.3: Changes in concentration of ethyl lactate during sixteen weeks of storage, at 5°C (◆), 15°C (■) and 25°C (△).

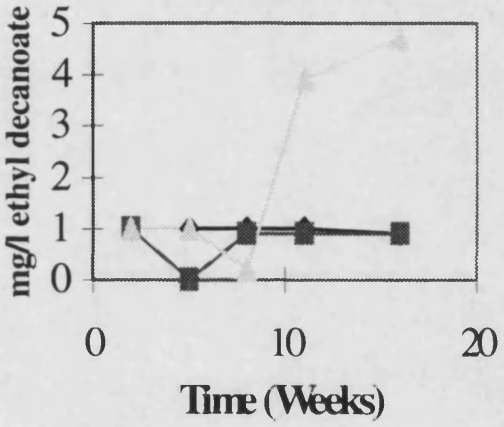

Figure 5.4: Changes in concentration of ethyl decanoate during sixteen weeks of storage, at 5°C (◆), 15°C (■) and 25°C (△).

Figure 5.4 shows that at the two lower temperatures, ethyl decanoate concentrations remained constant throughout storage, however, at 25°C, the concentration of this ester increased almost five-fold.

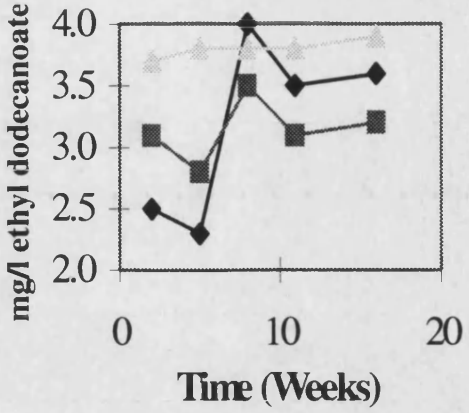

Figure 5.5: Changes in concentration of ethyl dodecanoate during sixteen weeks of storage, at 5°C (◆), 15°C (■) and 25°C (△).

Initial concentration of ethyl dodecanoate was not available, but by backwards extrapolation might be supposed to be approximately 3.2 mg/l. Figure 5.5 shows that after an initial reduction, ethyl dodecanoate levels increased more at lower temperatures than at higher temperatures, but stabilised at a lower level.

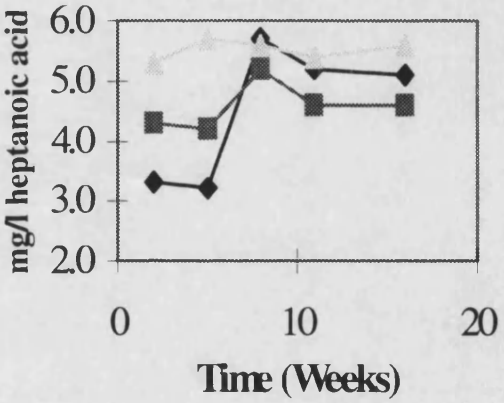

Figure 5.6: Changes in concentration of heptanoic acid during sixteen weeks of storage, at 5°C (◆), 15°C (■) and 25°C (△).

Once more, starting concentrations of this compound were unavailable. Concentration of heptanoic acid remained stable throughout storage at 25°C, but increased at the lower storage temperatures, as shown in Figure 5.6. An overall increase in concentration of 2-phenyl ethanol was observed at all temperatures, however, the rate of change was markedly slower at 5°C than at the two higher storage temperatures. There was also an increase in octanal and ethyl decanoate at the higher storage temperatures.

After sixteen weeks of storage, sensory analysis of cider stored at 5°, 15 ° and 25 °C was performed.

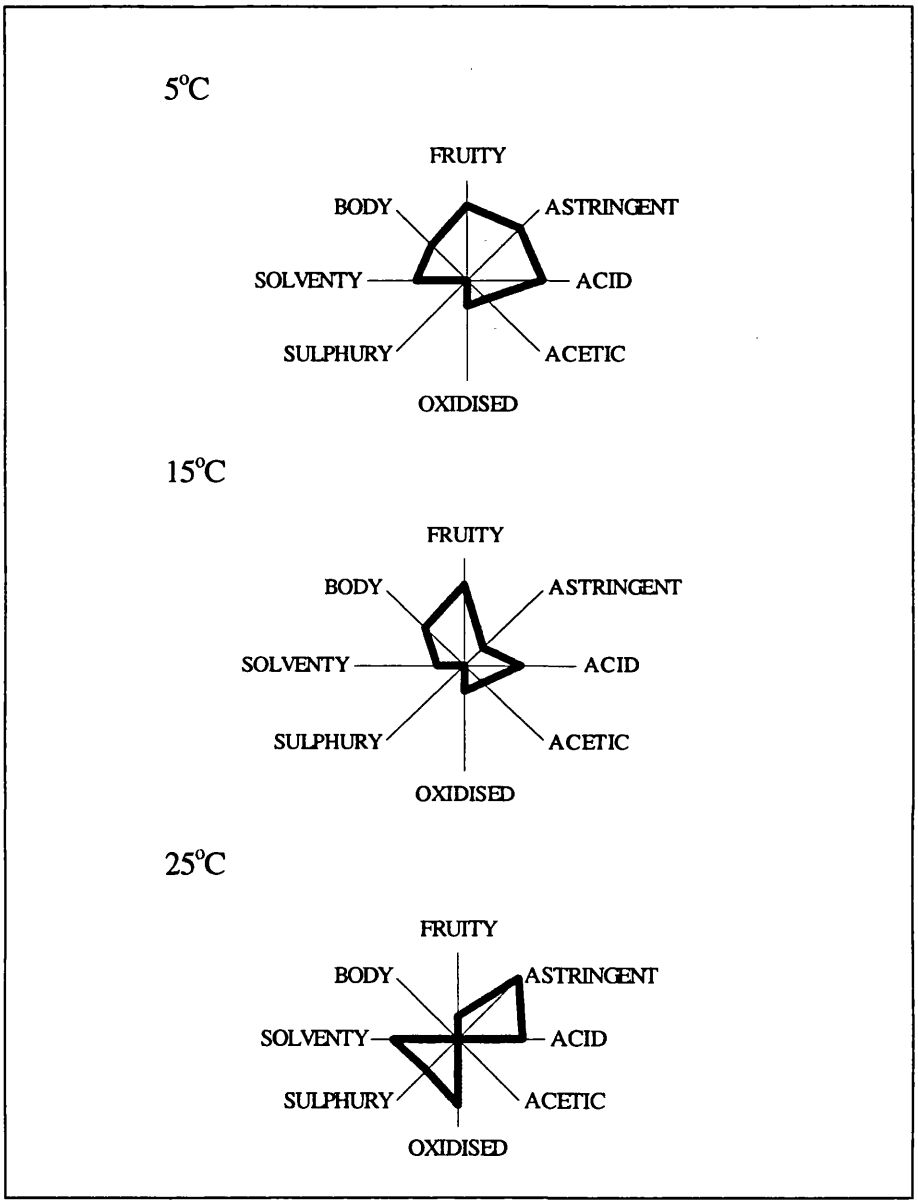

Figure 5.7: Cider flavour spider diagram illustrating the comparison of perceived flavours of ciders after they had been stored for sixteen weeks at 5°, 15° and 25°C for sixteen weeks.

Figure 5.7 is a diagrammatic representation of the sensory evaluation results. In addition, the stated preference was cider stored at 15 °C, then at 5 °C. Cider stored at the highest temperature was said to be bitter, burnt, sulphurous and very acidic.

5.2.1.2 Temperature #2

Laboratory cider, which had not undergone malo-lactic fermentation, was transferred into sterile glass containers after microfiltration (Section 3.2.5). Microbiological analysis on the cider showed a pure population of fermentation yeast (Table 5.3 and Appendix I). There was no change in microbial numbers by the end of storage at 5°C, a slight increase at 15°C, owing to increased cell growth and a decrease at 25°C, presumably due to increased autolysis.

Table 5.3: Micro-organisms present in 0.2 µm microfiltered, laboratory cider at beginning and end of storage trial at three different temperatures.

STORAGE TEMPERATURE	*Saccharomyces* spp. LOG AVG. CFU/ML IN CIDER	
	AT START OF STORAGE	AT END OF STORAGE
5°C	2.2	2.4
15°C	2.0	2.7
25°C	2.3	1.7

There was no change in concentration of the majority of flavour compounds during storage at any of the three temperatures, as shown in Table 5.4. 2-Methyl-1-butanol decreased at all three temperatures. Several compounds (*iso*-amyl acetate, diethyl succinate, ethyl dodecanoate and 4-ethyl phenol) increased only at 15°C. Other compounds appeared to change concentration due to temperature.

Hexanal, nonanal and ethyl decanoate decreased in concentration at higher storage temperatures, while heptanoic acid, ethyl lactate, ethyl benzoate and 2-phenyl ethanol increased in overall concentration during storage at higher temperatures.

Table 5.4: Overall effects of storage temperature on flavour compounds in concentrate-derived cider.

COMPOUND	5°C	15°C	25°C
hexanal	=	-	-
octanal	=	=	=
nonanal	=	=	-
undecanal	=	=	=
heptanoic acid	=	+	+
octanoic acid	=	=	=
2-methyl-1-butanol	-	-	-
hexanol	=	=	=
iso-amyl acetate	=	+	=
hexyl acetate	=	=	=
ethyl-2-methyl butyrate	=	=	=
ethyl hexanoate	=	=	=
ethyl lactate	=	+	+
ethyl octanoate	=	=	=
ethyl decanoate	=	=	-
diethyl succinate	-	+	-
ethyl dodecanoate	=	+	=
ethyl benzoate	=	=	+
phenylethyl acetate	=	=	=
2-phenyl ethanol	=	+	+
4-ethyl phenol	+	=	+

5.3.2 Effects of Filtration\Pulp Particle Size on Cider Flavour during Storage

5.3.2.1 Filtration #1

Cider, from a laboratory fermentation of fresh apple juice, was transferred to sterile containers and microbiological analysis was performed. The results of this analysis (Table 5.5) showed a mixed population, composed of micro-organisms from all four groups.

Micro-organisms in unfiltered cider declined to undetectable levels during storage, while micro-organisms in post fermentation filtered cider increased with the exception of lactic acid bacteria in post fermentation microfiltered cider, where none of these bacteria were detected after storage. All but non-*Saccharomyces* yeast declined to undetectable levels in pre- and post-filtered ciders. The wild yeast population increased during storage.

Table 5.5: Micro-organisms present in laboratory cider at beginning (pre-) and end (post-) of storage trial after three different filtration treatments. *Sacc* - *Saccharomyces* spp., non-*Sacc.* - non-*Saccharomyces* yeast, LAB - Lactic Acid Bacteria, AAB - Acetic Acid Bacteria.

	MICROBIAL COUNTS (LOG AVG. CFU/ML)							
	Sacc		non-*Sacc.*		LAB		AAB	
Storage	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
Unfiltered	>2.5	<0.7	>2.5	<0.7	>2.5	<0.7	>2.5	<0.7
Microfiltered	1.1	2.9	2.4	3.4	2.4	<0.7	2.0	3.0
Ultrafiltered	<0.7	2.5	1.8	1.8	1.5	1.5	1.9	1.9

Table 5.6: Overall effects of pre-storage filtration on flavour compounds in cider.

COMPOUND	Unfiltered	Micro-filtered	Ultra-filtered
hexanal	- -	- -	-
octanal	+	=	=
nonanal	=	=	=
undecanal	=	=	=
heptanoic acid	-	=	+
octanoic acid[1]	-	=	=
2-methyl-1-butanol	=	=	+
hexanol	- -	=	+
***iso*-amyl acetate**[2]	+	+	+
hexyl acetate	=	=	-
ethyl-2-methyl butyrate	-	-	=
ethyl hexanoate	=	=	+
ethyl lactate[3]	+ +	+ +	+
ethyl octanoate	- -	-	=
ethyl decanoate[4]	+ +	=	=
diethyl succinate	+ +	-	+
ethyl dodecanoate	-	-	+
ethyl benzoate[5]	=	=	+
phenylethyl acetate	=	+	-
2-phenyl ethanol	-	+	+
4-ethyl phenol	=	+	+

[1] see Fig.5.8; [2] see Fig.5.9; [3] see Fig.5.12; [4] see Fig.5.10; [5] see Fig.5.11.

Filtration had no discernible effects on concentrations of many compounds throughout storage (Table 5.6), but concentrations of octanoic acid, heptanoic acid and ethyl dodecanoate decreased as the degree of filtration increased, as illustrated by octanoic acid in Figure 5.8.

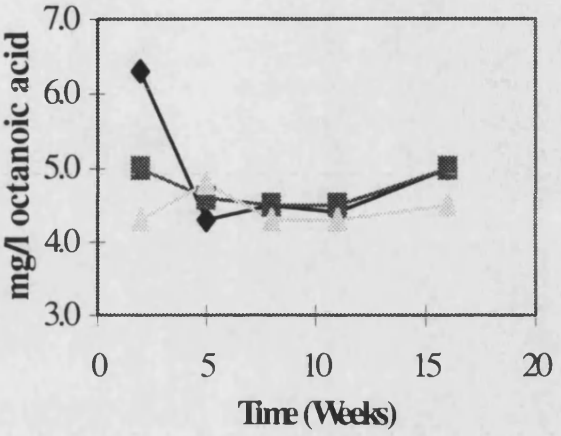

Figure 5.8: Changes in concentration of octanoic acid during sixteen weeks of storage, after no filtration (◆), microfiltration after fermentation (■), and ultrafiltration after fermentation (△).

Some volatile flavour compounds detected in this study appeared to be influenced by pre-storage filtration. Figures 5.9 to 5.12 illustrate some of the more complex effects of filtration on maturation flavour.

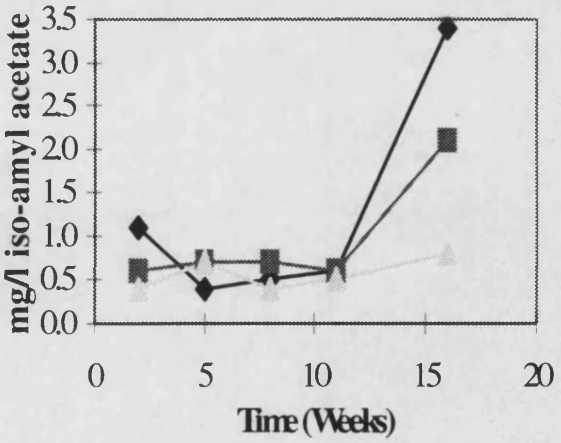

Figure 5.9: Changes in concentration of *iso*-amyl acetate during sixteen weeks of storage, after no filtration (◆), microfiltration after fermentation (■) and ultrafiltration after fermentation (△).

Initial concentrations of *iso*-amyl acetate in all the ciders were between 0.4 and 1.1 mg/l. This compound was detected at similar levels for the first eleven weeks of storage, after which the concentration increased. The greatest increase in concentration occurred in the unfiltered cider and the least increase occurred in the ultrafiltered cider (Figure 5.9).

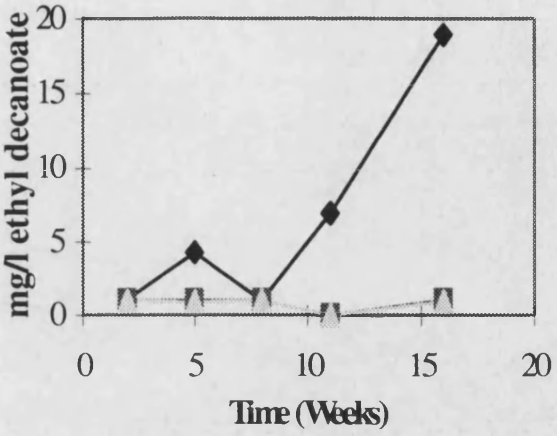

Figure 5.10: Changes in concentration of ethyl decanoate during sixteen weeks of storage, after no filtration (◆), microfiltration after fermentation (■) and ultrafiltration after fermentation (△).

Type and time of filtration had no effect on the concentration of ethyl decanoate, as shown in Figure 5.10. The two filtered ciders remained constant for the duration of storage. This was not, however, the situation for unfiltered cider, in which the initial concentration was much the same as in the other ciders, but a rapid increase was observed, with no apparent stabilisation even at the end of storage.

Unfiltered cider and post fermentation microfiltered cider showed no change in concentration of ethyl benzoate throughout storage. Cider that had undergone a prefermentation filtration stage and the post fermentation, ultrafiltered cider exhibited an increase in concentration of this compound after five weeks of storage.

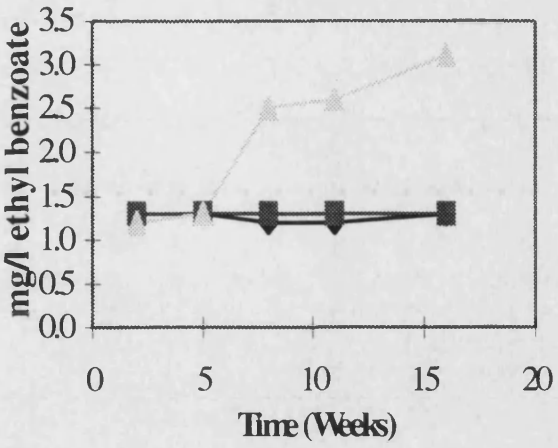

Figure 5.11: Changes in concentration of ethyl benzoate during sixteen weeks of storage, after no filtration (◆), microfiltration after fermentation (■) and ultrafiltration after fermentation (△).

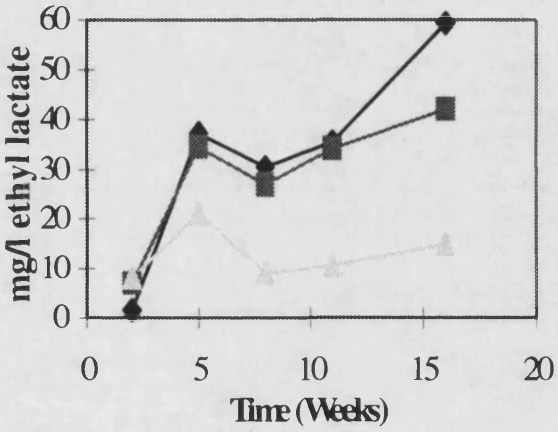

Figure 5.12: Changes in concentration of ethyl lactate during sixteen weeks of storage, after no filtration (◆), microfiltration after fermentation (■), ultrafiltration after fermentation (△).

Figure 5.12 illustrates the manner in which ethyl lactate profiles vary according to filtration. Ciders commenced storage with approximately the same concentration of this compound. All ciders showed a peak in ethyl lactate concentration at the fifth week of storage, which declined, then gradually rose again. The concentrations at the end of storage reflected the degree of filtration performed prior to storage. Unfiltered cider had the highest final concentration, then post fermentation, microfiltered cider.

5.2.2.2 Filtration #2

Filtered cider, derived from a laboratory fermentation of concentrated apple juice, was transferred to sterile containers and microbiological analysis showed a pure *Saccharomyces* population (Table 5.7).

Table 5.7: Micro-organisms present in laboratory cider at beginning and end of storage trial after three different filtration treatments.

FILTRATION TREATMENT	*Saccharomyces* spp. LOG AVG. CFU/ML IN CIDER	
	AT START OF STORAGE	AT END OF STORAGE
Unfiltered	4.1	3.1
Microfiltered	3.3	3.1
Ultrafiltered	<0.7	2.8

Fermentation yeast in unfiltered cider declined during storage, while they showed no change in microfiltered cider. They were, however, detectable in ultrafiltered cider after storage although none had been isolated from that cider at the start of storage.

Some of the compounds detected in the second investigation into the effects of filtration on cider flavour components were unaffected by filtration or subsequent storage, as shown in Table 5.8.

Initial concentrations of hexanal, octanal, heptanoic and octanoic acids, 2-methyl-1-butanol, ethyl dodecanoate, ethyl lactate, ethyl benzoate, 2-phenyl ethanol and 4-ethyl phenol were different according to their filtration treatment. Hexanol and nonanal were only detected in cider that had been ultrafiltered. In all cases, there was a greater concentration of compound the smaller the pore size of filtration, thus the concentrations of these compounds were least in unfiltered cider. This is contrary to the accepted hypothesis that filtration treatments result in a loss of aroma, due to the binding of small molecules, such as aroma compounds, to macromolecules, which are then removed by filtration (Pollard *et al.*, 1966; Pollard *et al.*, 1967; Klingshirn *et al.*, 1987; Voilley *et al.*, 1990).

Table 5.8: Overall effects of pre-storage filtration on flavour compounds in concentrate-derived cider.

COMPOUND	Unfiltered	Micro-filtered	Ultra-filtered
hexanal	+	+	+
octanal	=	=	=
nonanal	=	=	=
undecanal	=	=	=
heptanoic acid	-	=	-
octanoic acid	=	-	=
2-methyl-1-butanol	+	-	-
hexanol	=	=	=
iso-amyl acetate	=	=	=
hexyl acetate	=	=	=
ethyl-2-methyl butyrate	+	+	-
ethyl hexanoate	=	=	=
ethyl lactate	+	+	++
ethyl octanoate	=	=	=
ethyl decanoate	=	=	=
diethyl succinate	=	=	+
ethyl dodecanoate	=	-	-
ethyl benzoate	=	=	-
phenylethyl acetate	=	-	=
2-phenyl ethanol	+	-	-
4-ethyl phenol	=	-	-

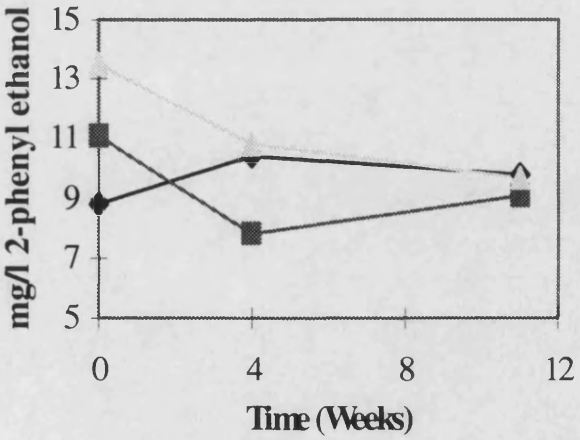

Figure 5.13: Change in concentration of 2-phenyl ethanol during eleven weeks of storage, after no filtration (◆), microfiltration after fermentation (■) and ultrafiltration after fermentation (△).

Prior to storage, the concentration of 2-phenyl ethanol was greatest in ultrafiltered cider (Figure 5.13). During storage, however, the concentration of this compound in each cider became similar.

5.3.3 Effect of Aeration and Anaerobiosis on Cider Flavour during Storage

5.3.3.1 Aeration/Anaerobiosis #1

Malo-lactic fermentation had occurred in this commercial cider, prior to racking off the lees. The cider was then microfiltered using a 0.45 µm filter. Carbon dioxide was bubbled through one cider sample until the dissolved oxygen was zero. The second cider sample was aerated until the dissolved oxygen content was 5%.

Microbiological analysis showed a mixed population, composed of micro-organisms from all four groups, each at similar levels (Table 5.9).

Table 5.9: Micro-organisms present in commercial cider at outset of storage trial in the presence or absence of oxygen.

MICROBIAL GROUP	LOG AVG. CFU/ML IN CIDER AT START OF STORAGE
Saccharomyces spp.	5.6
Non-*Saccharomyces* yeast	5.3
Lactic Acid Bacteria	5.5
Acetic Acid Bacteria	5.4

Table 5.10: Overall effects of carbon dioxide or oxygen additions to cider during storage on flavour compounds.

COMPOUND	CO_2	O_2
hexanal	+	+
octanal	+	-
nonanal	=	=
undecanal	=	=
heptanoic acid[1]	+	=
octanoic acid	-	-
2-methyl-1-butanol	++	++
hexanol	-	-
iso-amyl acetate	=	=
hexyl acetate	=	-
ethyl-2-methyl butyrate	=	=
ethyl hexanoate	=	=
ethyl lactate	-	+
ethyl octanoate	=	-
ethyl decanoate	=	=
diethyl succinate	-	-
ethyl dodecanoate	=	-
ethyl benzoate	+	+
phenylethyl acetate	+	+
2-phenyl ethanol	=	++
4-ethyl phenol	=	+

[1] see Figure 5.14.

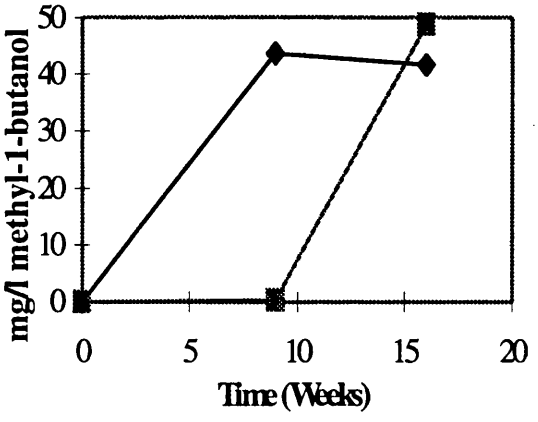

Figure 5.14: Changes in concentration of 2-methyl-1-butanol during sixteen weeks of storage, under carbon dioxide (◆) and oxygen (■).

Overall increases in concentration of hexanal, 2-methyl-1-butanol and ethyl benzoate appeared to be similar in both aerated and carbon dioxide charged ciders, the changes occurred more rapidly in cider stored under anaerobic conditions, as illustrated by 2-methyl-1-butanol in Figure 5.14.

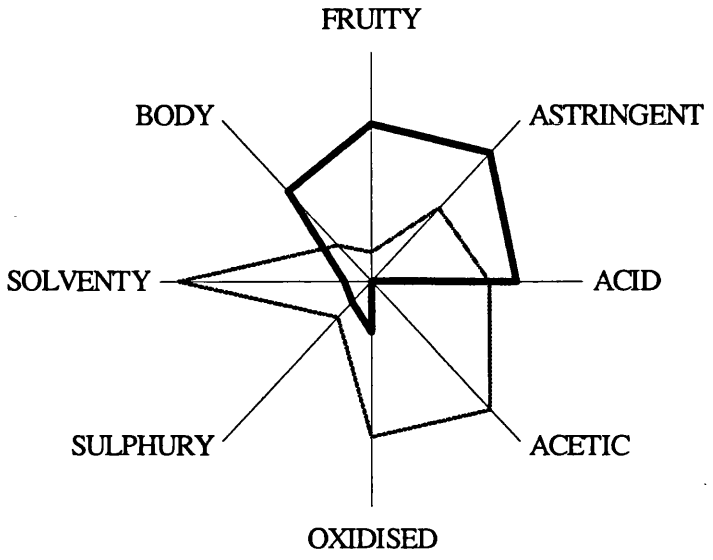

Figure 5.15: Cider flavour spider diagram illustrating the comparison of perceived flavours of cider stored in the presence of carbon dioxide (———) and oxygen (———) after sixteen weeks of storage.

Sensory analysis of cider that had been stored in the presence of carbon dioxide or oxygen for sixteen weeks was performed. Figure 5.15 is a diagrammatic representation of the sensory evaluation results. In addition, the stated preference was cider stored in the absence of oxygen. Cider stored in oxygen was said to be acetic, solventy and oxidised.

5.3.3.2 Aeration/Anaerobiosis # 2

A population of pure *Saccharomyces* spp. was detected in both ciders at a level of 1.4×10^2 cells/ml at the outset of storage.

Comparison of data from GC analysis on samples from the start of the investigation and after five weeks storage are shown in Table 5.11.

Table 5.11: Overall effects of carbon dioxide, air or oxygen additions to cider during storage, on flavour compounds.

COMPOUND	CO_2	O_2
hexanal	- -	-
octanal	=	=
nonanal	-	-
undecanal	=	=
heptanoic acid	-	-
octanoic acid	-	=
2-methyl-1-butanol	- -	-
hexanol	-	-
iso-amyl acetate	=	=
hexyl acetate	+	=
ethyl-2-methyl butyrate	-	=
ethyl hexanoate	=	-
ethyl lactate	+	+
ethyl octanoate	=	=
ethyl decanoate	+	-
diethyl succinate	=	=
ethyl dodecanoate	-	=
ethyl benzoate	=	=
phenylethyl acetate	-	+
2-phenol ethanol	=	+
4-ethyl phenol	=	=

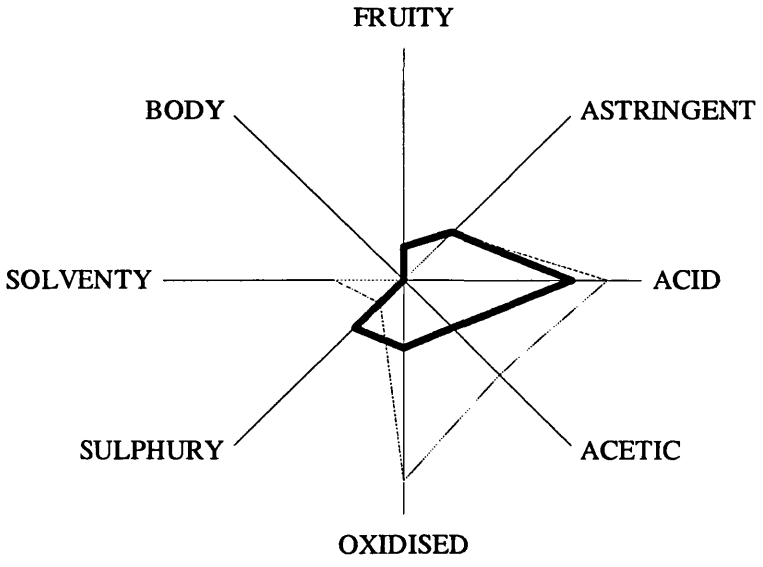

Figure 5.16: Cider flavour spider diagram illustrating the comparison of perceived flavours of cider stored in the presence of carbon dioxide (———) and oxygen (———).

Sensory analysis of ciders that had been stored for five weeks in the presence of either an excess of carbon dioxide or oxygen was performed. Figure 5.16 is a diagrammatic representation of the sensory evaluation results. No preference was stated for any of these ciders as both were considered atypical of storage strength cider. As before the aerated cider was described as acetic and oxidised.

5.3.4 Effect of Agitation on Cider Flavour during Storage

There were no detectable micro-organisms in the cider used for this investigation.

Recirculation of cider increased the concentration of phenylethyl acetate and decreased the concentrations of octanoic acid, hexanol, ethyl-2-methyl butyrate and ethyl hexanoate.

Table 5.12: Overall effects of recirculating cider during storage, on flavour compounds.

COMPOUND	Static	Circulating
hexanal	++	=
octanal	++	++
nonanal	+	+
undecanal	=	=
heptanoic acid	+	+
octanoic acid	+	=
2-methyl-1-butanol	--	--
hexanol	+	=
iso-amyl acetate	+	+
hexyl acetate	-	-
ethyl-2-methyl butyrate	+	-
ethyl hexanoate	++	=
ethyl lactate	-	-
ethyl octanoate	+	++
ethyl decanoate	+	+
diethyl succinate	+	+
ethyl dodecanoate	=	=
ethyl benzoate	=	=
phenylethyl acetate	-	+
2-phenyl ethanol	+	=
4-ethyl phenol	+	+

After three weeks of storage, sensory analysis of cider that was static or circulating throughout storage was performed. Figure 5.17 is a diagrammatic representation of the sensory evaluation results showing little variation in perceived flavour of ciders stored under static or recirculating conditions.

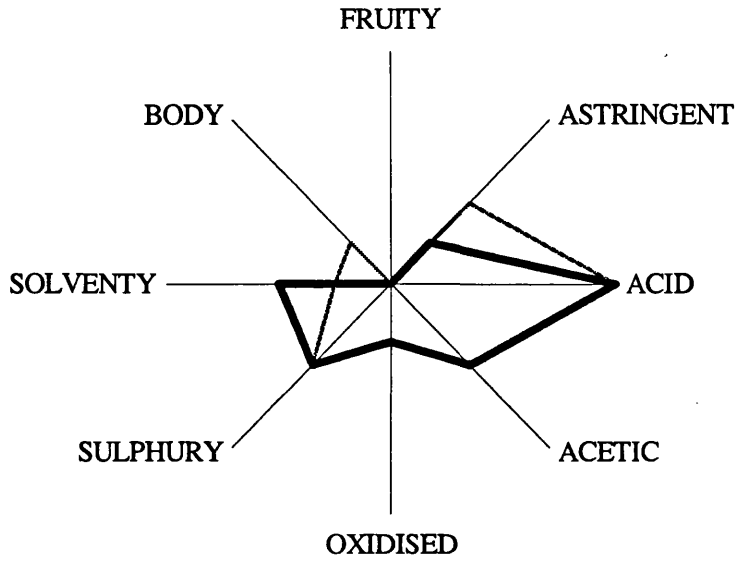

Figure 5.17: Cider flavour spider diagram illustrating the comparison of perceived flavours of cider stored under static (——) or circulating (——) conditions.

5.4 DISCUSSION

Schreier (1979) reported that only a few experimental results were available on the chemical processes that occur during wine storage, while Beech and Carr (1977) reported that nothing was yet known about chemical changes taking place during the period of cider storage. Very little additional information on changes in volatile flavour compounds during storage of cider has been reported.

Alcoholic beverages contain many minor compounds that appear after fermentation. This maturation appears to be necessary to improve flavour (Nykanen, 1986). Chemical changes, at this time, are complex, as changes induced at one stage may easily be reversed in subsequent steps (Williams, 1989). Fermentation flavour can be altered by post fermentation treatments, such as clarification, blending and stabilisation process (Williams, 1989). In wines, the concentration of higher boiling point compounds generally increase due to the evaporation of low boiling point components. The main ageing factor is

chemical equilibration. Hydrolysis and esterification bring about equilibration of esters and their constituent alcohols and acids. Thus, hexyl acetate decreases, while ethyl octanoate, ethyl decanoate and ethyl lactate increase (Williams, 1989). Etievant and Williams (1984) reported a decrease in ethyl butyrate and hexyl acetate by hydrolysis in the ageing of wines, while ethyl lactate increased, resulting from the esterification of lactic via pyruvate reduction with NADH and lactate dehydrogenase (Williams and May, 1981). In wines, a decrease in free fatty acids and tannins by chemical processes during storage was observed (Schreier, 1979).

Ciders stored under comparable conditions were examined for similarities. Firstly, a comparison between commercial fresh juice-derived cider that had undergone malo-lactic fermentation and a laboratory fermented cider that was also fresh juice derived, but had not undergone malo-lactic fermentation was made. Two thirds of the volatile compounds showed dissimilar concentration changes overall. Laboratory fermented cider initially contained greater concentrations of heptanoic acid, octanoic acid, *iso*-amyl acetate, ethyl-2-methyl butyrate, ethyl octanoate, ethyl dodecanoate and 2-phenyl ethanol and lower concentrations of ethyl lactate and diethyl succinate, in particular, than commercially fermented cider. Lower concentrations of the last two compounds may be attributed to the lack of malo-lactic fermentation, which is associated with increases in these two compounds. At the end of storage, however, the laboratory fermented cider contained more compounds in higher concentrations than the commercial cider, stored under the same conditions. Initial differences may be attributable to less evaporation of volatile compounds in commercial fermented cider, owing to the lower surface area to volume ratio and the occurrence of malo-lactic fermentation, which changes the volatile composition of cider. These differences in initial concentrations influence chemical equilibration during storage.

When commercially fermented, fresh juice-derived cider, microfiltered and stored under carbon dioxide was compared with laboratory fermented cider that had been microfiltered, similarities were observed. Two thirds of the volatile compounds showed dissimilar concentration changes overall. Commercial cider contained more compounds at higher concentrations than laboratory fermented cider, again attributable to lower losses of volatiles by carbon dioxide evolution in large scale fermentations. Concentrations of

compounds became more similar in the two ciders by the end of storage. Chemical equilibration is the most probable explanation for these changes during storage.

Comparisons between both laboratory fermented, concentrate-derived cider, micro-filtered and stored at 15°C, illustrated the problems associated with the sample preparation method used to examine the volatile components in cider by GC. The two ciders in this study were derived from the same fermentation and treated identically. Nevertheless, there were more compounds present in higher concentrations in the cider used for "temperature" storage than in cider used for "filtration" storage. At the end of storage, the difference between these two samples was even more apparent. Preparation and storage conditions were alike, as was the microflora in each cider. Sample preparation and analysis by GC were the most probable source of differences in volatile compound concentration.

There was an overall decrease in compound concentration during cider storage when carbon dioxide was bubbled into cider compared with cider stored in a static manner. Laboratory fermented, concentrate-derived cider that was stored with carbon dioxide initially contained more compounds in higher concentration than similarly produced cider that was stored with no carbon dioxide. At the end of storage, however, the former cider had lower concentrations of volatile compounds. These changes were most probably due to loss of volatile compounds by bubbling carbon dioxide through the cider, i.e. gas entrainment.

Laboratory fermented, concentrate-derived cider that had been ultrafiltered was compared with a similar cider, though which carbon dioxide gas was bubbled throughout storage. Overall changes in volatile compounds were more striking in cider through which carbon dioxide was bubbled. By comparison, the other cider contained more compounds in higher concentrations. The effects of sparging carbon dioxide through cider appear to be detrimental to the volatile composition of cider.

The final two comparable ciders were both laboratory fermented, concentrate derived ciders statically stored at 15°C after ultrafiltration. The cider in these two studies were from different fermentations, which may have given rise to the initial variations in concentrations of volatile components. Initial concentrations of many components were greater in the

cider for "filtration" studies than in cider for "recirculation" studies, but by the end of storage, lower concentrations of many volatile compounds were observed in the cider for "filtration" studies. A combination of errors in sample preparation for GC analysis, fermentation variations and subsequent chemical equilibration may be responsible for differences between these two samples.

Temperature of fermentation is reported to be responsible for basic evaporation losses. In addition, increased temperature increases ethyl lactate formation (Cotterell and McLellan, 1986) and retention of `heady', aromatic esters, such as ethyl octanoate, 2-phenylethyl acetate and ethyl decanoate. Fruity esters, such as *iso*-amyl acetate and hexyl acetate are retained at lower fermentation temperatures (Killian and Ough, 1979).

In this study, the effects of temperature on storage were examined in apple juice-derived cider and concentrate-derived cider. In the situation of minimal particulate and microbial content, increased storage temperature led to increased concentrations of ethyl benzoate, ethyl lactate (Cotterell and McLellan, 1986) and 2-phenyl ethanol and decreased concentration of octanoic acid. A corresponding increase in octanal was not, however, observed, so this decrease was unlikely to be as a result of autoxidation (Nykanen, 1986), neither was there a discernible increase in ethyl octanoate, due to esterification (Williams, 1989). In addition, the lower the storage temperature, the slower and lower the increase in 4-ethyl phenol. These changes are most probably due to chemical equilibration, rather than microbial activity.

A more complex situation was observed when investigating the effects of storage temperature on apple juice-derived cider. This cider possessed a large, mixed microflora and a high particulate matter content. In addition, biochemical changes had already occurred, by way of malo-lactic fermentation. Again, ethyl lactate formation was seen to increase with higher storage temperatures. Ethyl lactate, however, remained constant for the duration of storage, after the initial increase. Chemical equilibration of this compound with its components may have been achieved rapidly. Increases in ethyl decanoate and 2-phenyl ethanol were also more rapid at higher storage temperatures. Increases in heptanoic acid and ethyl dodecanoate were favoured by lower temperatures. In this cider,

changes were more likely to be as a result of complex chemical and microbial interactions that were influenced by storage temperature.

The preference for cider stored at 15°C indicated a compromise between minimising flavour changes at low temperature and causing off flavours, and oxidised and burnt characteristics at a higher temperature.

Filtration or other treatments that stabilise wines are well known to result in aroma loss, as the macromolecules that are removed during processing strongly bind small aroma molecules, which are therefore removed along with the particulate material. During fermentation, it was observed that increased solids in the medium resulted in increased higher alcohol production, due either to a high level of air entrapment by larger particles, or increased phenoloxidase activity by smaller particles, which lowered the dissolved oxygen content of the medium (Klingshirn *et al.*, 1987). All these effects are reported to occur during fermentation. A decrease in hexanol and *iso*-amyl acetate in a model wine that had been clarified by tangential ultrafiltration was reported (Voilley *et al.*, 1990). Serrano *et al.*, (1988) reported the organoleptic quality of wines may be affected in the first month after ultrafiltration, but no difference was detected after a year or two.

Effects of filtration on flavour development during storage were assessed with both apple juice-derived cider and concentrate-derived cider. In the apple juice-derived cider, the degree of post fermentation filtration affected several compounds, such as octanoic and heptanoic acids, ethyl decanoate, ethyl octanoate, hexanol and hexanal. The greater the filtration treatment, the greater the decrease in concentration of each compound. This is most probably due to removal of small aroma molecules bound to the macromolecules that were removed during filtering. The concentration of such compounds remained stable for the duration of storage. The concentration of ethyl-2-methyl butyrate increased during storage of unfiltered cider, but declined in the filtered ciders.

Iso-amyl acetate, ethyl decanoate, ethyl lactate and ethyl benzoate concentrations were not affected initially by filtration, but increased during storage. While the concentration of ethyl benzoate increased in ultrafiltered cider, the other three esters, (*iso*-amyl acetate, ethyl

lactate and ethyl decanoate) increased only in the unfiltered cider. Microbiological changes may have affected cider flavour along with chemical processes.

A very different situation was observed when investigating the effects of filtration on concentrate-derived cider. Whereas increased filtration decreased the initial aroma concentration in apple juice-derived cider, the filtration of concentrate-derived cider appeared to cause an increase in concentration of many aroma compounds. The levels of heptanoic acid, octanoic acid, octanal, ethyl dodecanoate and 2-methyl-1-butanol increased initially and then remained stable for the rest of maturation. Ethyl benzoate, 2-phenyl ethanol and 4-ethyl phenol were also affected in this manner but, concentrations changed during storage, such that the same concentration for each compound was detected in each cider. It would appear that a stable level was achieved during storage, most probably by chemical equilibration.

Ultrafiltered cider showed increases in ethyl lactate and diethyl succinate concentration, but ethyl-2-methyl butyrate decreased during storage. Unfiltered and microfiltered ciders did not show any changes in concentration of these compounds during storage.

During fermentation, oxygen restricts the formation of *iso*-amyl acetate and ethyl hexanoate (Nykanen, 1986). Indeed, any aeration at that time reduces higher alcohol production (Beech, 1972b). During storage, oxygen contact with fermented beverages leads to undesirable changes in colour, flavour and bouquet (Jones *et al.*, 1986). Oxidation of alcohols, degradation of amino acids and autoxidation of fatty acids leads to the formation of aldehydes (Nykanen, 1986).

In apple juice-derived cider, the concentration of ethyl-2-methyl butyrate and 2-phenyl ethanol increased in the presence of oxygen, but otherwise remained stable in non-aerated cider. Ethyl benzoate, 2-methyl-1-butanol and hexanal also increase in concentration when cider is stored in the presence of oxygen. Concentrations, however, decreased when stored anaerobically. *Iso*-amyl acetate and hexyl acetate decreased in the presence of oxygen.

Volatiles in concentrate-derived cider exhibited very different chemical changes when stored in the presence of oxygen. Rather than increasing in concentration when stored in

the presence of oxygen, 2-methyl-1-butanol, ethyl-2-methyl butyrate and hexanal all decreased. Decreases in octanal concentration occurred in ciders derived from both apple juice and concentrate. Aldehydes, however, are normally expected to increase in the presence of oxygen.

Constant motion of cider during storage inhibited (or affected in some other manner) the increase in ethyl-2-methyl butyrate, hexanol, hexanal and ethyl hexanoate, which was observed to occur in statically stored cider. Phenylethyl acetate and ethyl octanoate increased when stored in agitated cider, while 2-phenyl ethanol concentration decreased.

5.5 SUMMARY

- Very little is yet known about chemical changes taking place during the period of cider storage.

- Preliminary investigations suggested that sterile fermented cider would exhibit minimal flavour changes during storage.

- Although storage temperature does not appear significantly to influence many aroma compounds in cider, sensory evaluation indicated that a mid-range storage temperature conferred the best flavour characteristics, organoleptically.

- Post fermentation filtration of cider derived from apple juice has a substantially greater effect on the initial concentration of aroma compounds than in a concentrate derived cider.

- Although the presence of oxygen during storage did not appear to affect many of the aroma compounds, sensory evaluation of these ciders indicate very profound, undesirable changes in flavour had occurred. Clearly, the compounds responsible for these changes were not detected. Storage of `sterile' cider with carbon dioxide had little effect on flavour compounds or sensory quality.

- Cider that has undergone malo-lactic fermentation, exhibits many more flavour changes, influenced by several factors, including temperature and filtration, than cider that has not undergone this secondary fermentation.

- Micro-organisms, even after malo-lactic fermentation, apparently influence cider flavour, adding to its complexity.

- Key compounds that may be used to indicate the degree of maturation include ethyl lactate, which increases during storage, and is influenced by temperature, filtration and oxygen content of cider. 2-Phenyl ethanol may also be considered, as it is readily

detected in cider and contributes to the typical cider aroma (along with phenylethyl acetate and hexanol).

- Ethyl-2-methyl butyrate and 2-methyl-1-butanol may also be potential cider maturation marker compounds, as they are present in cider in easily detected quantities, and are influenced by various storage parameters, including filtration and aeration.

CHAPTER SIX

MICROBIAL ECOLOGY OF STORAGE VATS

6.1 INTRODUCTION

Many alcoholic beverages are traditionally matured in casks of wood, such as oak and larch, when the beverage undergoes several changes. Oak wood, for example, can impart many organoleptically interesting substances to a beverage. Material is extracted from the wood and reactions between the beverage and wood derived components may occur (Chatonnet *et al.*, 1992; Piggott *et al.*, 1993; Clyne *et al.*, 1993). Repeated cask use is accompanied by significant reductions in wood components available for extraction. Oak casks are, therefore, frequently replaced in wine and spirit manufacture. The situation is different in the English cider industry. Oak wood vessels used for cider storage generally have a greater capacity (100 thousand gallons/454600 l) than casks (30-50 gallons/136-228 l) used in wine and spirit industries (Chatonnet *et al.*, 1992). They have also been used repeatedly for at least forty years compared with an average of six years in the wine industry. The influence of wood extracts on cider maturation flavour is, therefore likely to be of little consequence.

It is, however, recognised that changes in cider flavour during storage varies from vat to vat. Microbiological differences between vats are a probable reason for these variations in flavour. The porous structure of wood provides an ideal environment for entrapment and subsequent colonisation of cider micro-organisms (Fredette, 1970). Biofilms are thought to serve as reservoirs for both beneficial and spoilage micro-organisms in the cider industry. Persistence of these micro-organisms throughout cider production has significant implications for flavour development and control during storage, both in traditional wood vats and the modern, stainless steel vessels.

The ability of micro-organisms to persist in wood, even after cleaning, is substantiated within other areas of the food industry (Abrishami *et al.*, 1994; Ak *et al.*, 1994). Micro-organisms on and within the surface of storage vessels may contribute to the complex

microflora of cider, and thus to the final product flavour. An understanding of how these entrapped micro-organisms influence cider flavour is important to retain existing product flavour, as wood vats are replaced with vessels constructed from food grade, sterilisable materials.

Variations in indigenous, colonising micro-organisms between storage vessels were investigated by swabbing a small area of several vats, before and after cleaning. In this way, the distribution and persistence of yeast and bacteria throughout production cycles of emptying, cleaning and filling could be monitored. (Vats are routinely cleaned with a ball head sprayer - first washed with 0.5% sodium carbonate at 60°C for 20 minutes, then rinsed for a further 20 minutes with water at the same temperature).

In addition, microbial penetration, colonisation and rate of succession of vat wood was determined by suspending cubes of unused vat oak in storage cider. Visualisation of these micro-organisms was accomplished with the use of scanning electron microscopy (SEM). The effect of these colonising micro-organisms on cider flavour was also examined.

6.2 EXPERIMENTAL

6.2.1 Microbial Colonisation of Oak Wood

A block of unused English oak was first planed to remove any surface dirt. It was then cut into 2.5 cm sided cubes and the ends cut across the grain of wood were sealed with varnish. Nylon thread was used to fasten each cube onto a length of stainless steel rod and the whole apparatus was then autoclaved in distilled water to ensure sterility. This apparatus was then suspended in the cider 1 metre below the surface. Each week, for ten weeks, a cube of suspended wood was removed from the cider and prepared for examination by SEM. A cube that had not been suspended in cider was also prepared and viewed by SEM to confirm the initial absence of any micro-organisms in the wood.

6.2.2 Processing of Wood for Scanning Electron Microscopy

Taking care not to allow the wood to dry out, the outer surface of each collected wood cube was sliced with a sharp blade, to yield sections of less than 1 mm in thickness. Similar sections were removed from the centre, by cleaving the cube in half. Each wood section

was placed in a vial containing 1 ml of 2% gluteraldehyde in 1/4 strength Ringer's solution. The gluteraldehyde fixative was carefully removed after 1 hour and the section washed twice for 20 minutes with 1 ml aliquots of 1/4 strength Ringer's solution. 1 ml of 1% osmium tetroxide in 1/4 strength Ringer's solution was then used as a second fixative agent. After 1 hour, the osmium tetroxide was removed and the wood section washed twice for 20 minutes with 1 ml aliquots of 1/4 strength Ringer's solution. The prepared wood sections were then dehydrated in three stages using 30, 50 and 70% acetone before freeze drying overnight. Finally, the sections were mounted and gold coated for six minutes (Edwards Splutter Coater SISOB) before examination with the SEM (Jeol JSM T330).

6.2.3 Influence on Cider Flavour of Colonising Micro-Organisms

An oak cube, suspended for ten days in laboratory fermented storage cider was rinsed with 1/4 strength Ringer's solution and resuspended for 30 days in 2.5 l filtered (0.2 μm) laboratory fermented cider at 20°C. A similar volume of cider without the wood cube inoculation was set up as a control. Organic acid, flavour profiles and microbial contents of the ciders were observed before and after resuspension of the cube.

6.2.4 Persistence of Colonising Micro-Organisms throughout the Production Cycle

An area approximately 25 cm^2 of the side or base of six epoxy resin lined, concrete vessels and five wooden vats, used for cider storage, were swabbed using moistened alginate swabs, both before and after cleaning. Each swab was then dissolved in 10 ml Calgon Ringer's solution. A 10-fold serial dilution, with 1/4 strength Ringer's solution, to 10^{-5} was performed on each of these suspensions and 0.1 ml aliquots of each were spread plated in duplicate onto WLNA, WLD, Raka Ray and Lysine Agars (Appendix I).

Effects of cleaning on microbial populations entrapped within oak wood were assessed by scrubbing oak cubes with water at 60°C or by washing with 0.1 M sodium hydroxide at 40°C. After cleaning, sections from were obtained from the cube and prepared for SEM.

6.3 RESULTS

6.3.1 Microbial Colonisation of Oak Wood

Oak wood cubes that had not been suspended in cider were found to be free of micro-organisms. Wood penetration, adhesion and colonisation by cider micro-organisms are illustrated in Figures 6.1 to 6.10. Both the surface and centre (12 mm depth) of wood cubes suspended in storage cider were examined by SEM.

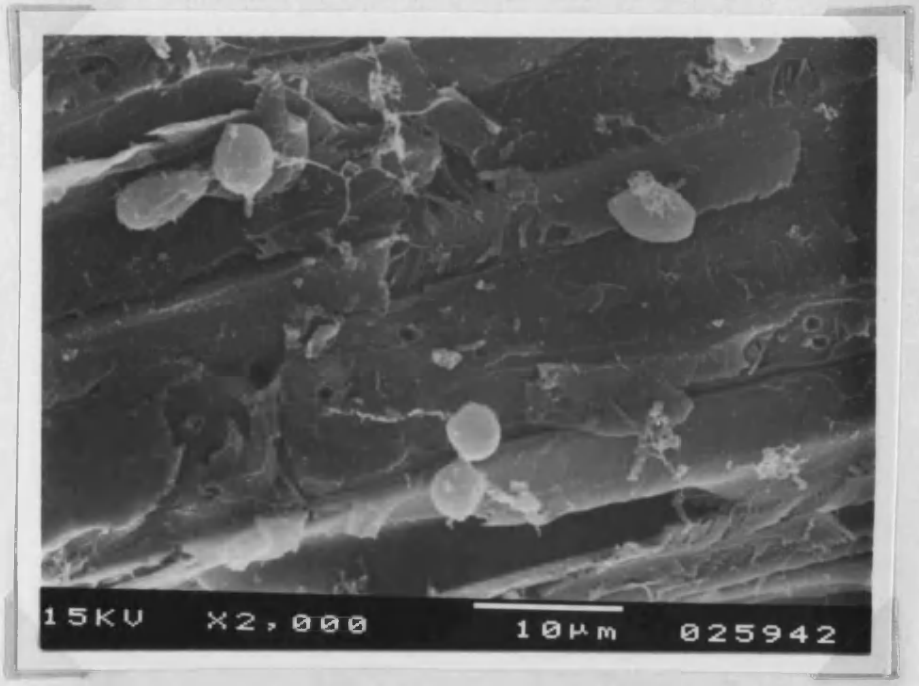

Figure 6.1 Section 2.5 mm from surface, from wood suspended in cider for two weeks.

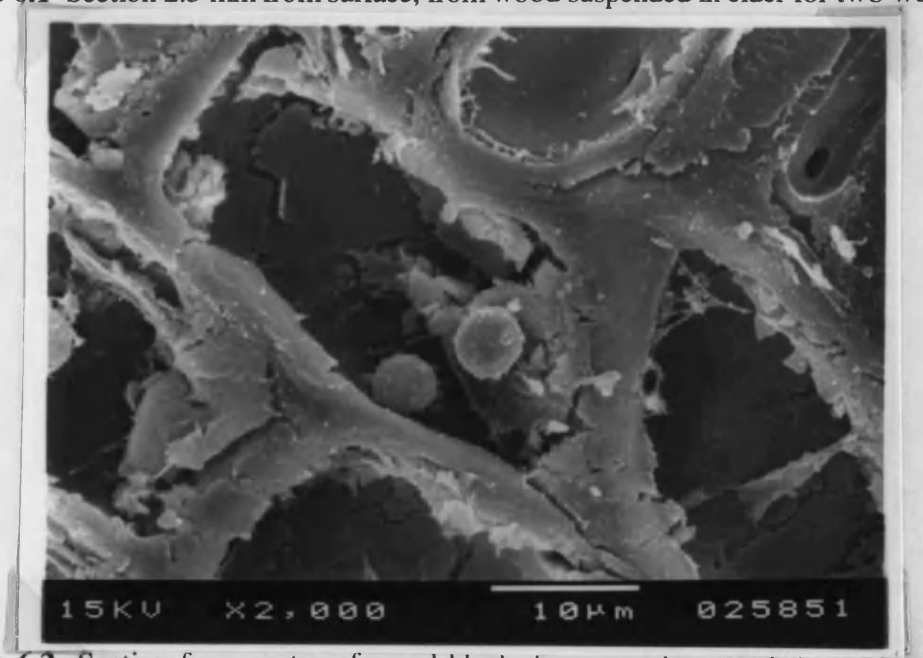

Figure 6.2 Section from centre of wood block, from wood suspended in cider for two weeks.

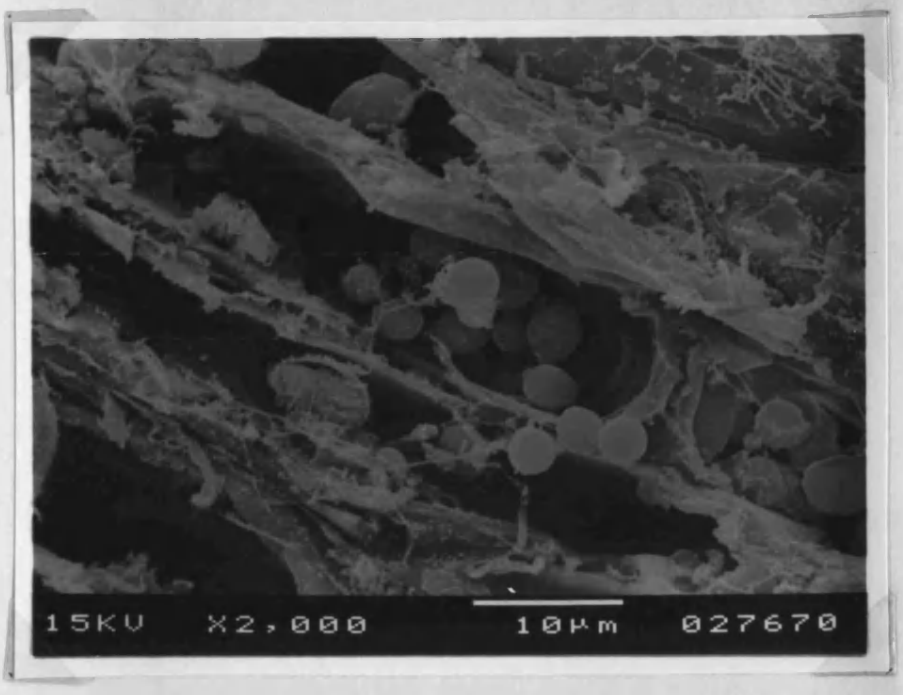

Figure 6.3 Section 2.5 mm from surface, from wood suspended in cider for four weeks.

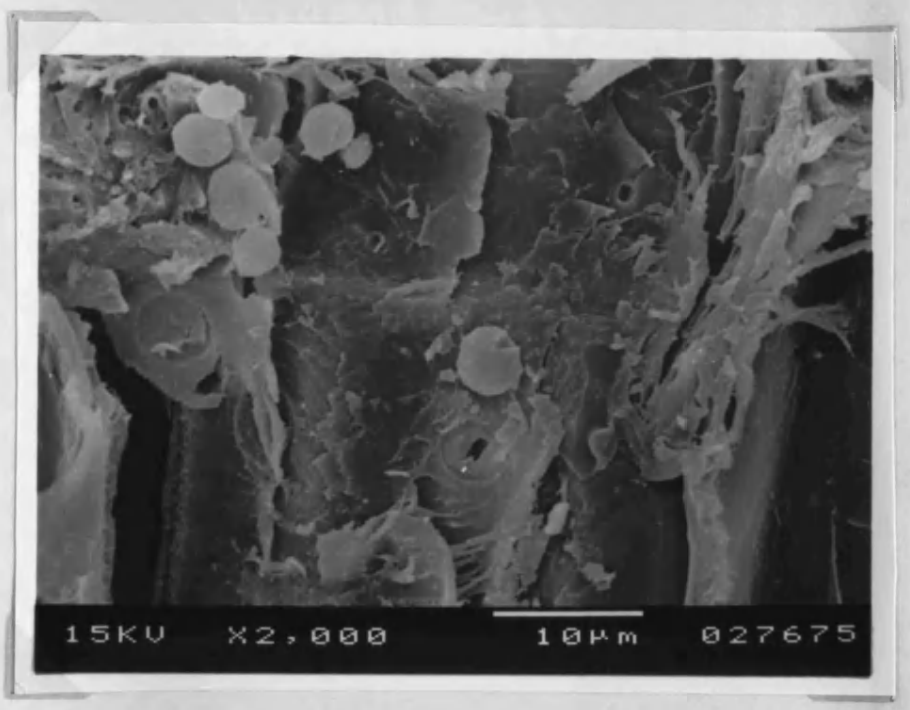

Figure 6.4 Section from centre of wood block, from wood suspended in cider for four weeks.

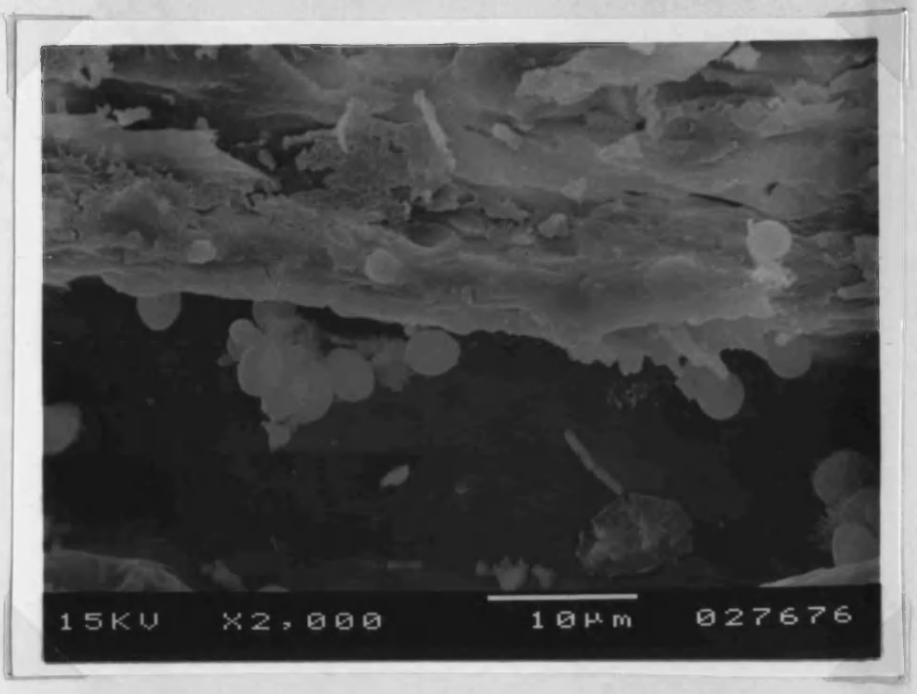

Figure 6.5 Section 2.5 mm from surface, from wood suspended in cider for five weeks.

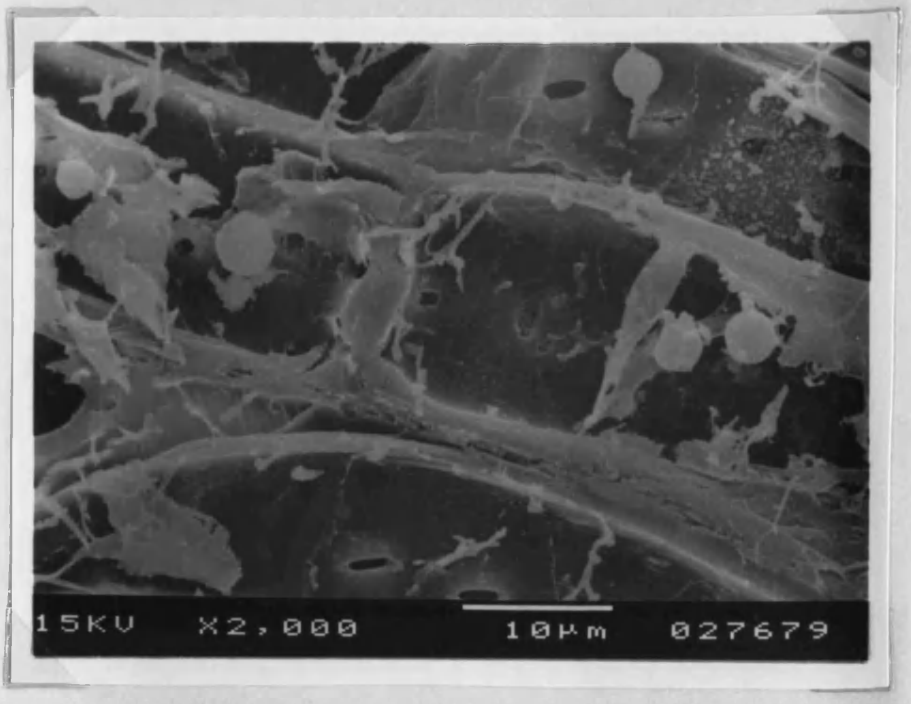

Figure 6.6 Section from centre of wood block, from wood suspended in cider for five weeks.

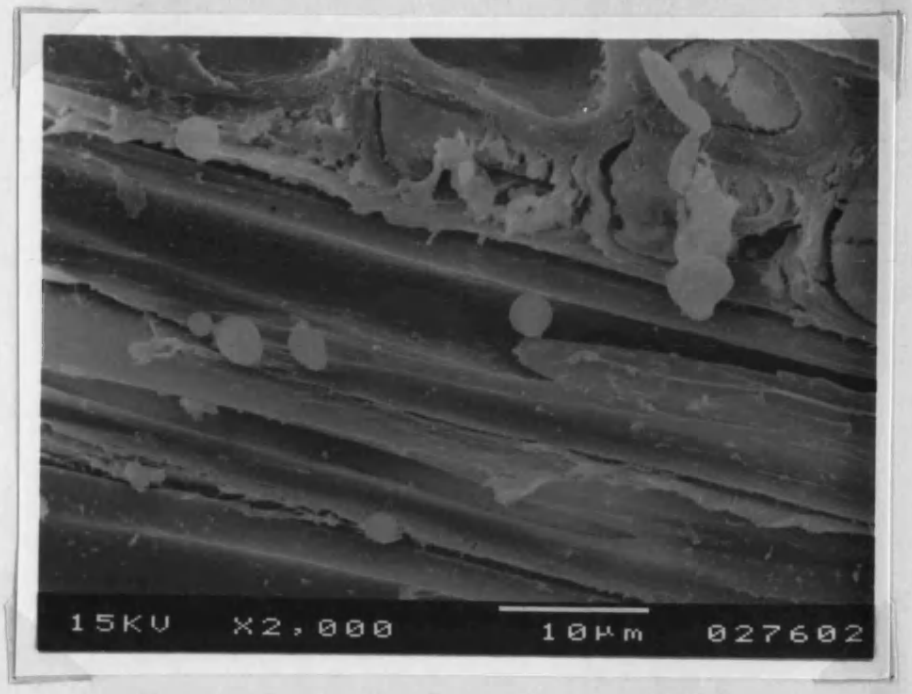

Figure 6.7 Section 2.5 mm from surface, from wood suspended in cider for nine weeks, showing both *Saccharomyces* and non-*Saccharomyces* yeast.

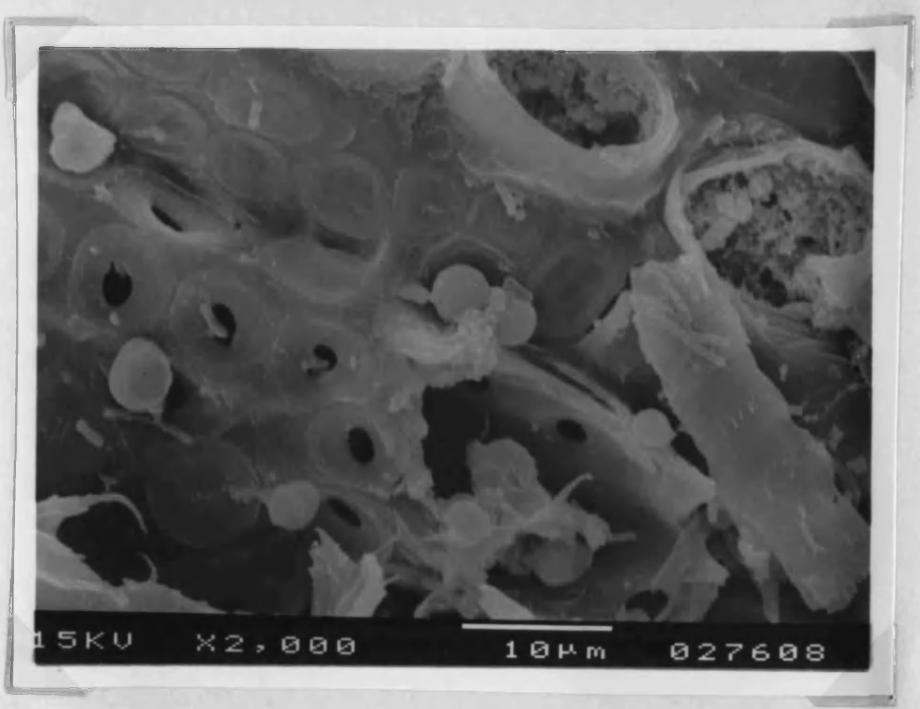

Figure 6.8 Section from centre of wood block, from wood suspended in cider for nine weeks, showing both yeast and lactobacilli.

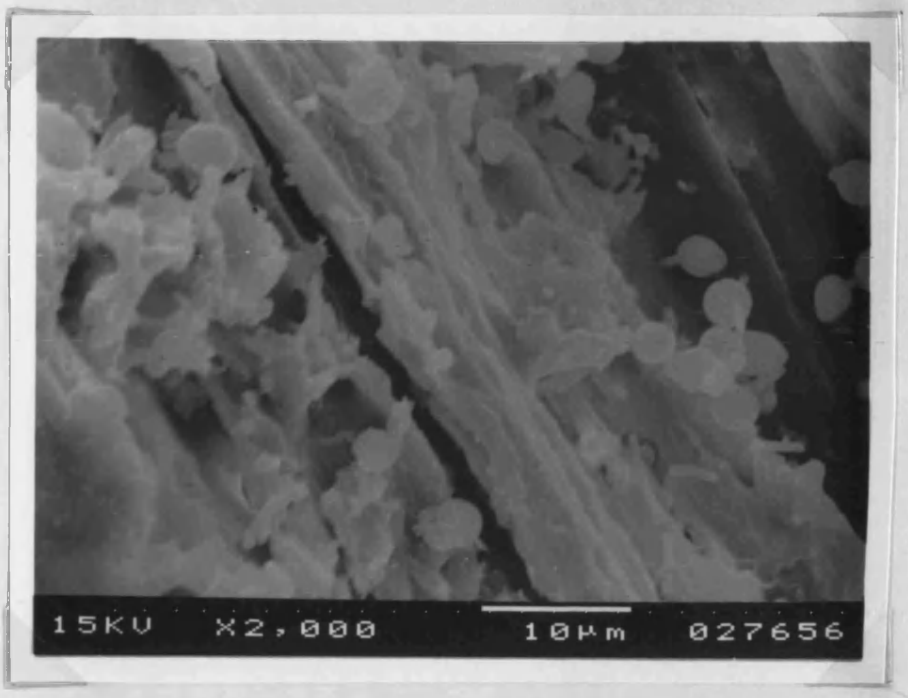

Figure 6.9 Section 2.5 mm from surface, from wood suspended in cider for ten weeks.

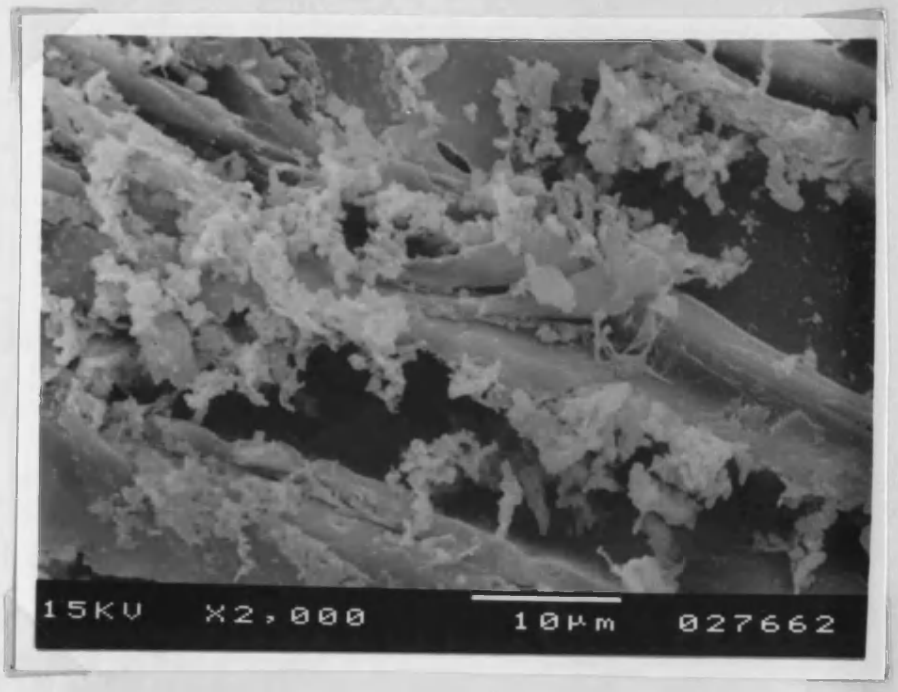

Figure 6.10 Section from centre of wood block, from wood suspended in cider for ten weeks.

Yeast and bacteria had rapidly penetrated the wood structure, even to a depth of 1.2 cm, within two weeks of suspension in cider. Yeast cells shown in Figures 6.1 and 6.2 were already irreversibly attached to the rough surfaces of the wood. Yeast growth and division just beneath the surface were apparent by week four (Figure 6.3), although further into the wood structure, colonisation processes had not yet progressed beyond attachment.

A week later, yeast colonies were established and growing beneath the surface, as can be seen by the budding cells in Figure 6.5. Lactobacilli are also visible as unique cells, adhering to wood, rather than associated with yeast. Colonisation at a depth of 1.2 cm into the wood had not greatly progressed from the previous week, although some penetration and adherence of lactobacilli had occurred. Figure 6.6 also illustrates improved attachment on rough surfaces.

Figures 6.7 and 6.8 depict a different structure of the vascular system in wood, as the large, open pores in previous micrographs are absent. Instead, there are very much smaller pits and longitudinal sections through the vascular system. There was much less entrapment and hence adhesion on these smoother, more open structures. Bacteria were more ingressive than yeast after nine weeks of suspension, but were less prolific. The presence of non-*Saccharomyces* yeast in wood was rarely evident, but unmistakable as seen in Figure 6.7. Bacterial adhesion and colonisation progressed rapidly within the wood. Clusters of lactobacilli and even a chain of cocci can be found in Figure 6.8.

Colonisation was well established throughout the wood by the tenth week of suspension. Yeast and lactobacilli remained closely associated as they encroached upon the less enclosed areas of wood, as observed in Figure 6.9. The final micrograph, Figure 6.10, illustrates the invasive nature of an exopolysaccharide forming coccal species, (e.g. *Leuconostoc mesenteroides*). Although slower to adhere to surfaces than yeast or lactobacilli, cocci present in cider formed a comprehensive biofilm over and across the vascular structures of wood, increasing entrapment of other micro-organisms. Exopolysaccharide production is very apparent in Figure 6.10.

6.3.2 Influence on Cider Flavour by Colonising Micro-Organisms

Although not sterile, the 2.5 l quantities of cider base used to investigate the effects on flavour of micro-organisms entrapped in wood contained *Saccharomyces* spp. to a level of 2.0×10^4 cfu/ml and non-*Saccharomyces* yeast and acetic acid bacteria at levels no greater than 1.0×10^2 cfu/ml. No lactic acid bacteria were isolated prior to inoculation with colonised wood or in the control cider after one month. A noticeable change in microflora was observed in the inoculated cider after one month, the most obvious being a noteworthy number of lactic acid bacteria, i.e. 2.0×10^5 cfu/ml, as seen in Figure 6.11. Although acetic acid bacteria numbers also rose, the increase was observed in the control cider as well as in inoculated cider.

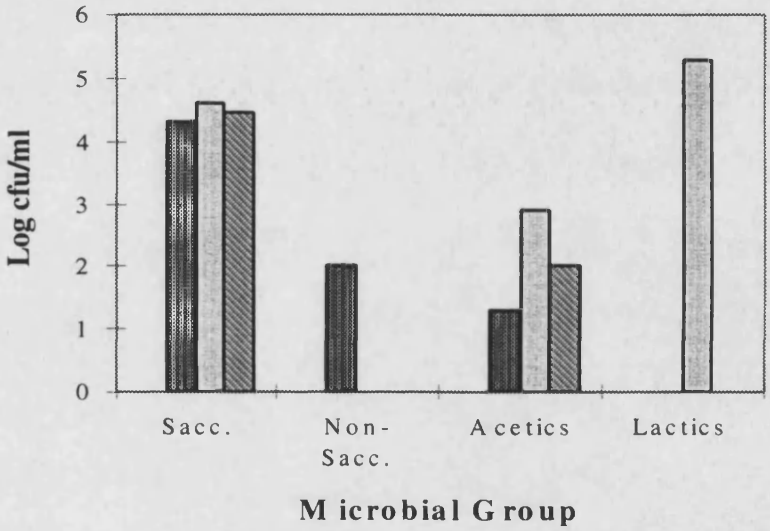

Figure 6.11 Microflora of cider before (■) and after (▩) inoculation with colonised wood block and without inoculation (▧), the last two, matured for 30 days at 20°C.

A film of yeast, presumptively *Candida* spp., and acetic acid bacteria developed within one week of inoculation over the surface of the cider. A similar film had also been observed on the surface of the commercial cider in which the wood blocks had originally been suspended.

While succinic and citric acid levels remained constant during one month's storage, acetic acid levels rose in both ciders. Malic and lactic acid levels changed significantly during one month storage of cider inoculated with a colonised wood block. Figure 6.12 illustrates the significant reduction of malic acid, while lactic acid content had dramatically increased.

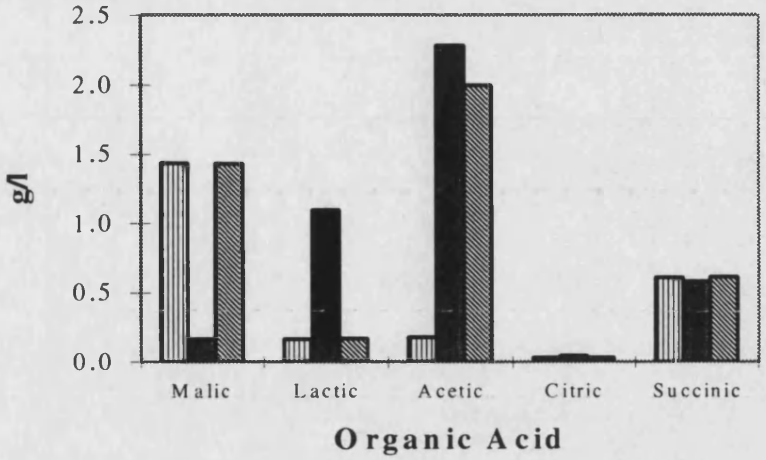

Figure 6.12 Organic acid profiles in cider before (▨) and after (■) inoculation with colonised wood block and without inoculation (▧), the last two matured for 30 days at 20°C.

Analysis of the cider before (Figure 6.13) and after storage (Figure 6.14) by gas chromatography (Section 3.4.1) illustrated the changes in flavour profile after one month storage in the presence of micro-organisms. Many additional compounds were detected in the matured cider as well as changes in concentration of existing compounds. Some compounds present in the base cider were no longer detected in matured cider.

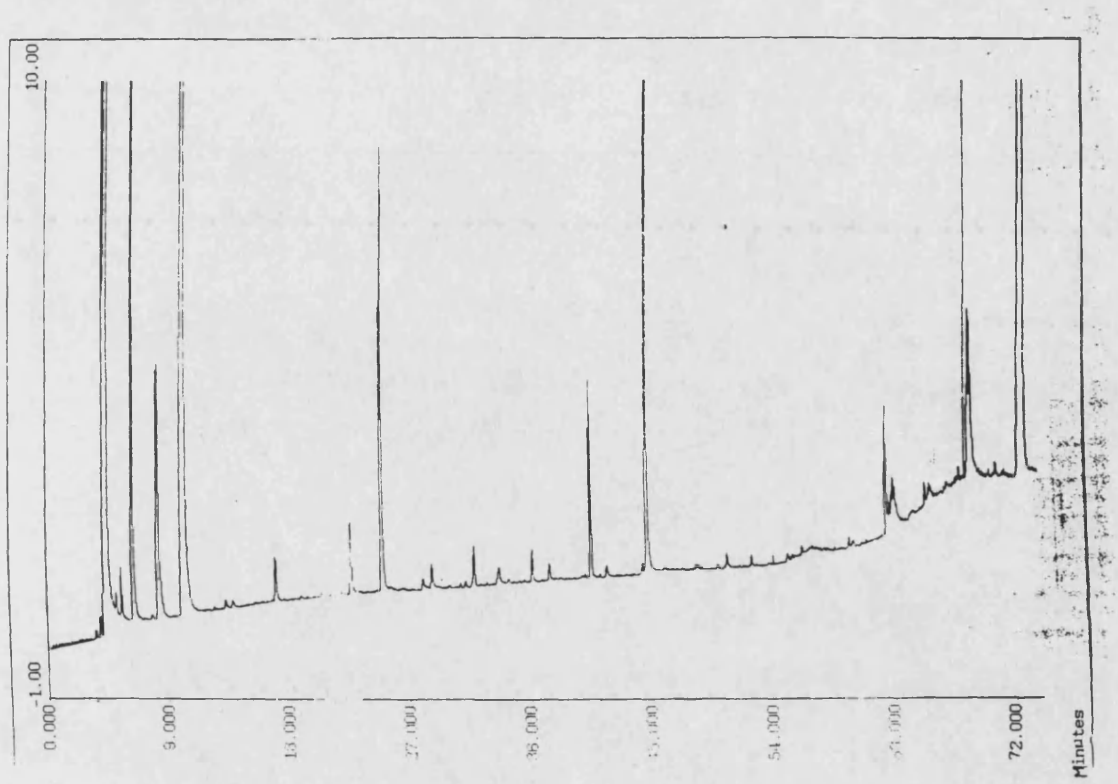

Figure 6.13 Gas chromatogram of cider before a 30 day maturation with wood colonising micro-organisms.

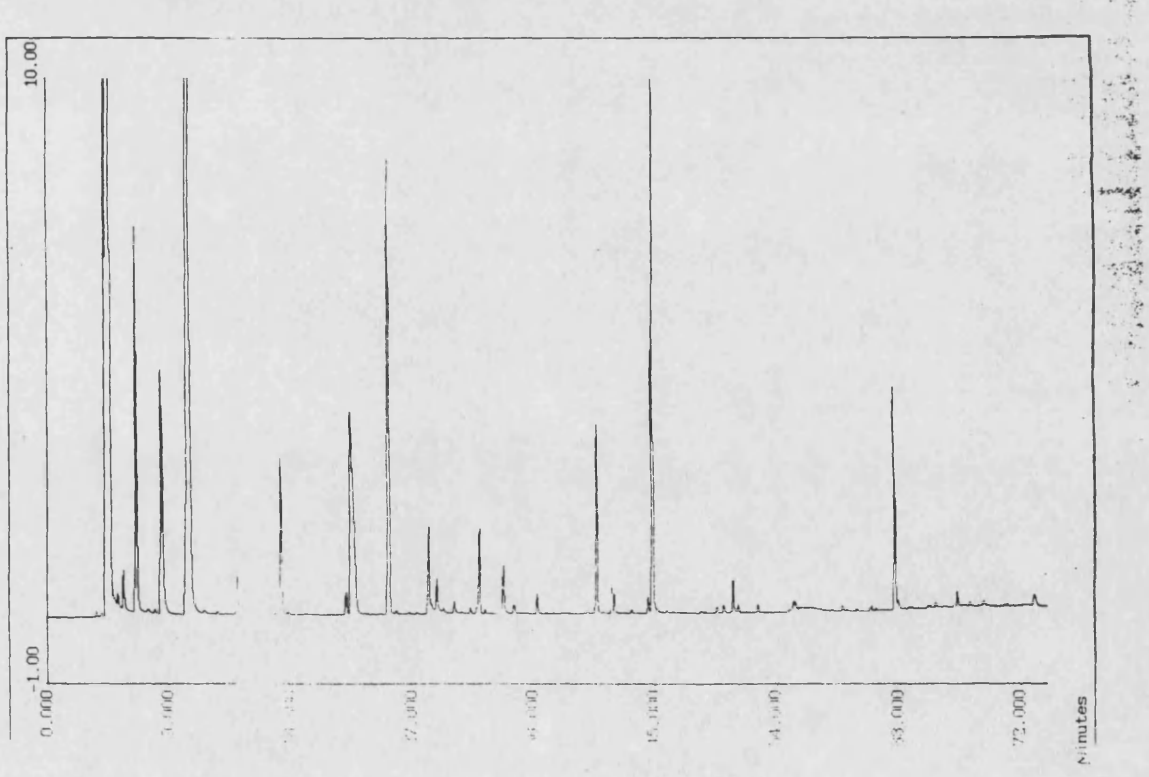

Figure 6.14 Gas chromatogram of volatile flavour components in cider after a 30 day maturation with wood colonising micro-organisms

Table 6.1 Volatile Flavour Compounds Detected in Cider, Before and After Inoculation for 30 Days with Wood Block Colonised with Cider Micro-Organisms

Peak No	Identity	Conc (mg/l)		% in extract	
		Before	After	Before	After
1		17.61	20.07	5.066	7.216
3		0.645	0.435	0.186	0.156
4	ethyl-2-methylbutyrate	2.013	1.699	0.579	0.611
5	hexanal	27.92	22.18	8.033	7.975
6	iso-amyl-acetate	0	0.335	0.000	0.120
7		0	0.2573	0.000	0.093
8	heptan-3-one	21	21	6.042	7.551
9	2-methyl-1-butanol,	189.3	84.2	54.462	30.274
10	ethyl hexanoate	0	0.2283	0.000	0.082
11		0	0.124	0.000	0.045
12	hexyl acetate	0.017	0.0726	0.005	0.026
13	octanal	0.541	2.843	0.156	1.022
14		0.033	0.0695	0.009	0.025
15		0	0.0352	0.000	0.013
16		0	0.0614	0.000	0.022
17	ethyl lactate	0	0.0375	0.000	0.013
18		0	0.0878	0.000	0.032
19	hexanol	3.113	9.64	0.896	3.466
20		0	0.176	0.000	0.063
21	nonanal	0.01	0.1437	0.003	0.052
22		0.013	0.0409	0.004	0.015
23		0.177	0.0518	0.051	0.019
24	ethyl octanoate	4.4	25.41	1.266	9.136
25		0.102	1.51	0.029	0.543
26		0	0.0698	0.000	0.025
27		0	0.0589	0.000	0.021
28		23.56	24.81	6.778	8.920
29		0.103	0.1745	0.030	0.063
30		0	0.1721	0.000	0.062
31		0	0.1424	0.000	0.051
32		0	5.83	0.000	2.096
33		1.477	2.271	0.425	0.817
34		0	0.423	0.000	0.152
35	undecanal	0.252	0.766	0.073	0.275
36		0	0.0364	0.000	0.013
37		0	0.0428	0.000	0.015
38		0	0.2659	0.000	0.096
39		0	0.2178	0.000	0.078
40	ethyl decanoate	2.4	4.42	0.690	1.589
41	ethyl benzoate	0	0.1652	0.000	0.059
42		0	0.0096	0.000	0.003
43	diethyl succinate	0.996	2.909	0.287	1.046
44		0	0.546	0.000	0.196
45		0.093	0.0505	0.027	0.018
46		0	0.0307	0.000	0.011
47		1.545	0.999	0.445	0.359
48		0	0.0658	0.000	0.024
49		0	0.0735	0.000	0.026
50		0.737	0.0317	0.212	0.011
51		0	0.1256	0.000	0.045
52		0	0.0559	0.000	0.020

Peak No	Identity	Conc (mg/l) Before	After	% in extract Before	After
53	phenylethyl acetate	0.319	0.1696	0.092	0.061
54	ethyl dodecanoate	9.35	9.26	2.690	3.329
55		0.098	0.0721	0.028	0.026
56		0.627	1.412	0.180	0.508
57		0	0.0227	0.000	0.008
58	2-phenyl-ethanol	0	0.1851	0.000	0.067
59		0.491	0.777	0.141	0.279
60	heptanoic acid	36.5	27.1	10.501	9.744
61		0	0.1317	0.000	0.047
62		0	0.0564	0.000	0.020
63		0	0.0226	0.000	0.008
64		0	0.1655	0.000	0.060
65	octanoic acid	0.596	0.0367	0.171	0.013
66		0	0.1823	0.000	0.066
67		0	0.372	0.000	0.134
68		0.948	1.709	0.273	0.614
69		0	0.367	0.000	0.132
70		0.545	0.0369	0.157	0.013
71		0	0.43	0.000	0.155
72	p-ethyl phenol	0.032	0.049	0.009	0.018
73		0	0.0249	0.000	0.009
74		0.014	0.0722	0.004	0.026

Figures 6.13 and 6.14, the chromatograms of volatile flavour components, extracted from cider before and after inoculation with wood colonised with cider micro-organisms showed apparent increases in component concentrations. These changes are more clearly illustrated in Table 6.1 where both increases in component concentrations and number of flavour components detected can be seen. Initially, only 35 components were detected in base cider, but after maturation, 73 volatile components were detected, even if only in trace amounts, for example, *iso*-amyl acetate, ethyl hexanoate, ethyl lactate and 2-phenyl ethanol. There was a decrease in lower boiling point compounds, such as ethyl-2-methyl butyrate and 2-methyl-1-butanol. Octanoic acid also decreased with maturation, with an increase in ethyl octanoate.

There was an increase in the aldehydes, octanal, nonanal and undecanal. Aldehydes have low threshold values, that is, they are detected organoleptically in low concentrations and are therefore important flavour compounds. Although there was a marked change in concentration of some compounds, for example ethyl-2-methyl butyrate, hexanal and heptanoic acid, the percentage in the extract was not significantly altered.

6.3.3 Persistence of Colonising Micro-Organisms throughout the Storage Cycle

Each vat swabbed in this investigation possessed a unique microflora. Three issues were examined: firstly, total number of micro-organisms in each of the four microbial groups isolated; secondly, the number of apparently different species in each group; thirdly, the differences in microbial populations of wood vats and lined concrete vessels (denoted W_n, C_n respectively). Figure 6.15 not only illustrates the different microbial numbers of each vat, but more importantly, how routine cleaning procedures affect these microbial numbers.

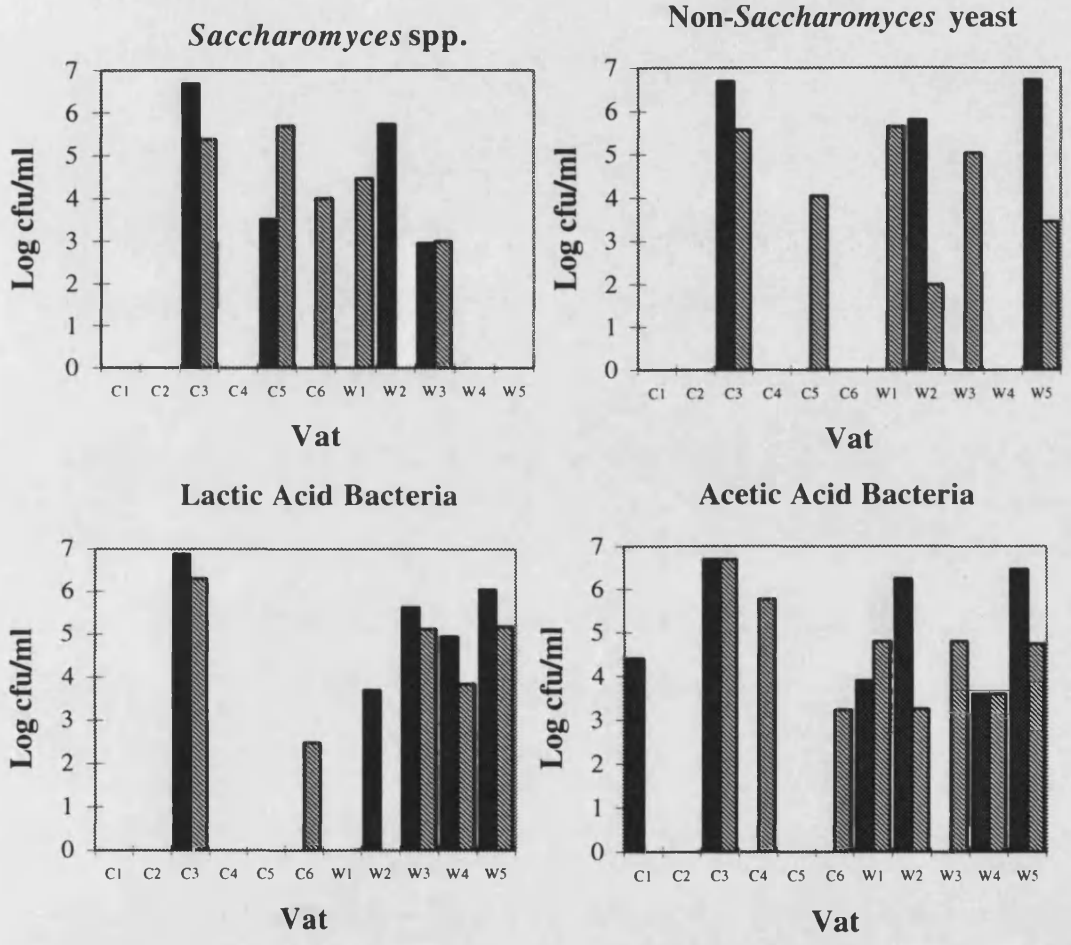

Figure 6.15 Total microbial numbers in vats, before (■) and after (■) cleaning.

Using the Wilcoxon Rank Sum Test (Miller and Miller, 1988), there was no significant difference ($P>0.05$) in microbial population numbers in either wood or lined concrete vats, pre- and post-cleaning.

Other than vat C3, which possessed an unusually large mixed population, concrete storage vessels were relatively free of microbial contaminants, even before cleaning. Wood vats,

however, were heavily contaminated with both lactic acid and acetic acid bacteria, and their numbers were not significantly affected by cleaning.

The number of presumptive species in each microbial group enumerated is shown in Figure 6.16. Different species were determined using colony and cell morphology (Appendix II), while also considering the isolation medium and conditions.

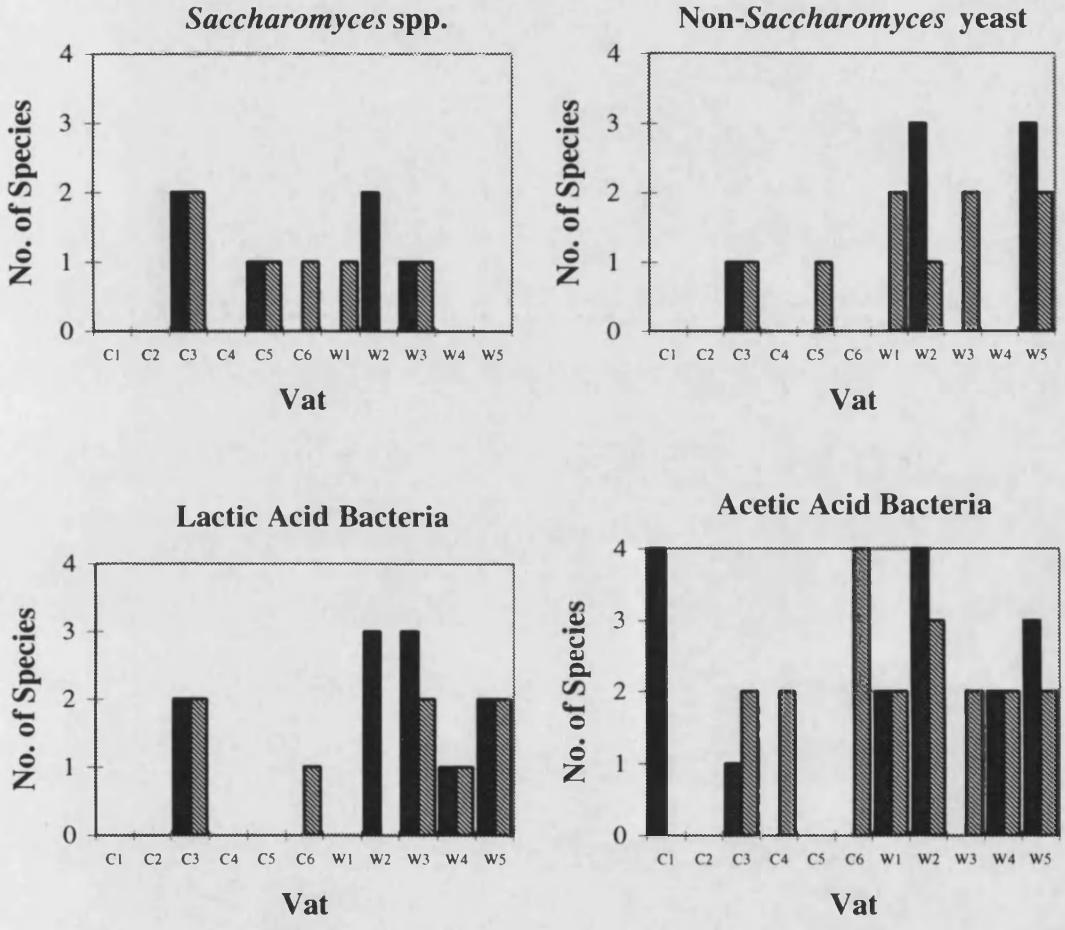

Figure 6.16 Number of species on storage vessel surface before (■) and after (■) cleaning.

Using the Wilcoxon Rank Sum Test (Miller and Miller, 1988), there was no significant difference ($P>0.05$) in the number of microbial species in both wood and lined concrete vats, pre- and post- cleaning. When changes were observed, there was either a reduction in the number of species isolated after cleaning, or the appearance of species after cleaning when none had been isolated previously, probably due to the mechanical action of cleaning.

Consequences of washing on microbial cells entrapped within vat wood 2.5 mm from surface, are illustrated with the following micrographs, Figures 6.17 to 6.19.

Figure 6.17 Microbial colonisation of oak wood after ten weeks suspension in storage cider.

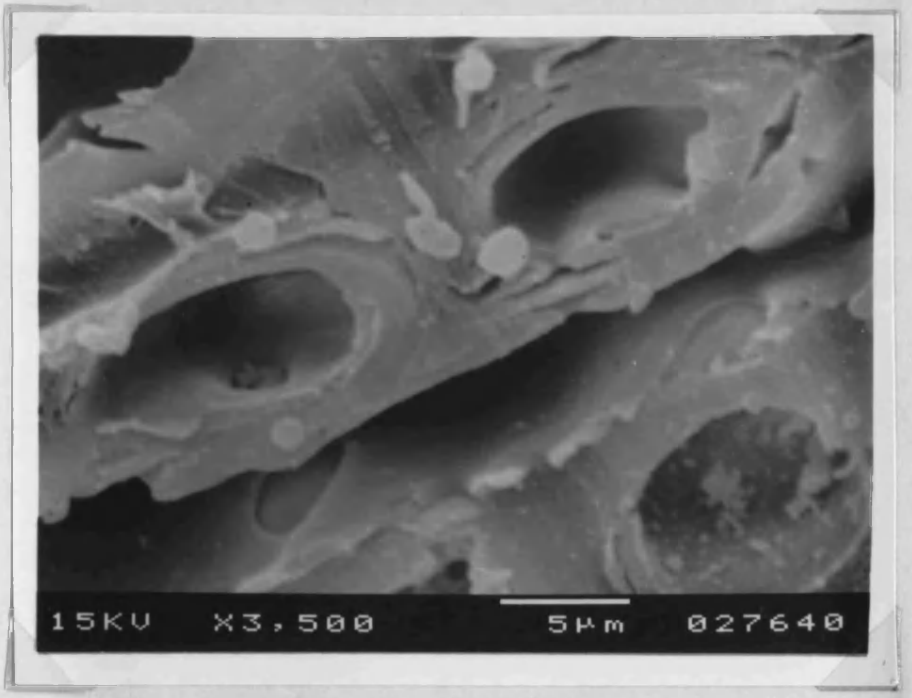

Figure 6.18 Microbial entrapment in oak wood after scrubbing with water held at 60°C.

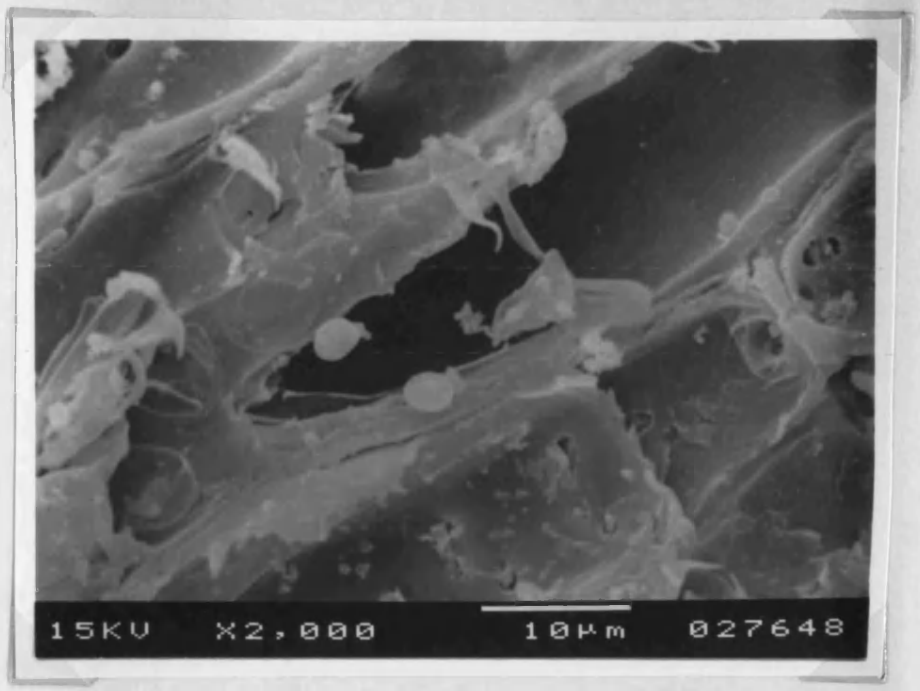

Figure 6.19 Extent of microbial entrapment in oak wood after cleaning with warm (40°C) 0.1 M sodium hydroxide.

Lactobacilli were strongly associated with yeast, as previously shown in Section 6.3.1. Microbial penetration and adhesion in wood were extensive after suspension in cider for ten weeks. Yeast colonisation and growth within the pores of the wood are clearly shown in Figure 6.17.

Microbial adhesion of micro-organisms in oak wood was apparently diminished, but not eliminated after cleaning. The remaining cells are of undetermined viability and vitality. Sufficient micro-organisms, however, appear to remain entrapped within the porous structure of the wood for subsequent growth and recolonisation to be feasible.

6.4 DISCUSSION

Within the food industry as a whole, it has been found that bacteria retained on and in wood are viable and metabolically active. They reside in the structural and vegetative elements of xylem tissues, and it has been speculated that the same water, food and mineral conducting

functions attributed to plant tissues are also the means of redistributing micro-organisms throughout wood (Abrishami *et al.*, 1994).

This investigation ascertained the rate and extent of wood penetration and colonisation by cider micro-organisms, and effects of cleaning on population distribution and density. This study also confirmed that changes in cider flavour were caused by these colonising micro-organisms.

Diffusion of cider and associated micro-organisms into the wood blocks was observed within the first week of this investigation. The porosity of wood, especially unused wood (Abrishami *et al.*, 1994) and fluid dynamic effects (Gilberts *et al.*, 1993) are known to encourage penetration and concentration of cells at a surface, the first stage of colonisation. In non-flowing systems (as found in a cider storage vat) aggregation, Brownian motion and chemotactic responses to nutrient gradients by cells can all favourably influence this initial stage of colonisation (Gilberts *et al.*, 1993) Next was reversible adhesion, reversed by rinsing (Carpentier and Cerf, 1993; Anon., 1994). This phase was not illustrated, as the wood blocks underwent several rinsing stages during preparation for SEM. Rinsing removes cells that are adhering to a surface by a combination of Van der Waal's and electrostatic forces (Characklis et al., 1989; Gilberts *et al.*, 1993). This explains the presence of micro-organisms on surfaces of both wood and concrete vats after washing, when few, if any, had been isolated before cleaning. Cells are detached from surfaces, or other cells, during rinsing and can be, therefore, isolated by swabbing.

Cells captured on the series of micrographs can be assumed to be irreversibly attached to surfaces, as they remain on the wood even after thorough rinsing. Irreversible adhesion is thought to be a result of chemical bonding and hydrophobic interactions between the cell and surface (Characklis *et al.*, 1989). Destructive procedures, such as scrubbing, illustrated by the washing procedures in Section 6.2.4, were required to remove cells from beneath the surface of wood, once irreversible adhesion had occurred. This has previously been noted for biofilms in general (Carpentier and Cerf, 1993) and wood in particular (Ak *et al.*, 1994). Micro-organisms attached to a surface are more resistant to environmental fluctuations and antimicrobial agent, and thus constitute a reservoir for future growth and activity (Carpentier and Cerf, 1993; Gilberts *et al.*, 1993). For example, environmental desiccation

favours exopolysaccharide production by micro-organisms (Carpentier and Cerf, 1993), while colonies exposed to feast/famine conditions accumulate to a higher extent than those with a continuous nutrient supply (Characklis *et al.*, 1989). This has particular implications in cider storage, as vats are constantly being emptied, cleaned and refilled, continually exposing the colonising micro-organisms to a changing environment, both physically and nutritionally.

Once attached, reproduction and growth of cells occurred, thereby promoting colonisation of surfaces. This stage of colonisation, as reported by Characklis and colleagues (1989) and Carpentier and Cerf (1993) can take no more than two hours, or may take days or months to attain a state of equilibrium. Cells at the centre of the wood blocks took longer to reach this growth stage, possibly due to nutrient limitation, or owing to a lower density of cells penetrating that depth of wood compared to the cell density just beneath the surface.

The smaller size of bacterial cells enabled noticeable penetration by lactobacilli into the centre of the block through the small pores or 'pits' of the vascular tissues, explaining the almost ubiquitous presence of lactic acid and acetic acid bacteria in wood vats, both before and after cleaning. Increased aeration with washing favours acetic acid bacteria, which grow rapidly on exposure to air.

The extent of colonisation increased with surface roughness, micro-organisms were consistently found to be concentrated in crevices as opposed to smooth, flat surfaces (Figures 6.7 and 6.8). This is illustrated by the generally less populated nature of concrete vats, even prior to cleaning, as the lined concrete vats have a relatively non-porous, smoother surface when compared with wood. Characklis and colleagues (1989) surmised that decreased desorption, lower shear forces and increased surface area available for adsorption were the reasons for increased colonisation with increased surface roughness. They suggested that it was a non-selective process as the colonies eventually spread across the whole surface. Once established, a biofilm undergoes a dispersive phase (Gilberts *et al.*, 1993). In part, this phase was illustrated by the appearance of lactic acid bacteria in significant numbers in cider inoculated with a colonised wood block. These lactic acid bacteria were responsible for the decarboxylation of malic acid to lactic acid, that is, malo-

lactic fermentation. Acetic acid levels might be attributed to the activities of acetic acid bacteria, heterofermentative lactic acid bacteria or direct oxidation of ethanol to acetic acid or a combination thereof. In essence, micro-organisms colonising wood vats do, to an extent, influence final product flavour.

6.5 SUMMARY

- Wood is readily penetrated and colonised by cider micro-organisms, initially by yeast, but more importantly, latter stages are dominated by bacterial colonisation.

- These colonising micro-organisms can be removed by destructive cleaning procedures, but nevertheless, a reservoir is maintained.

- Microbial populations in wood influence cider flavour. This is a complex issue, dependant upon distribution of types of micro-organisms and their interactions with one another and their environment.

- As wood vats are phased out of use and sterilisable stainless increasingly utilised, these findings have several implications. On one hand, final product flavour can be controlled to a greater extent, with the possible use of selected bacterial inoculation to create standard, desirable flavour changes to a product. On the other hand, the characteristic spicy, complex flavour may be replaced by a crisp, clean taste, with a subsequent loss of the traditional cider character.

CHAPTER SEVEN

SOME OBSERVATIONS ON MICROBIOLOGICAL CHANGES DURING CIDER MATURATION

7.1 INTRODUCTION

The microflora of freshly pressed apple juice has been found to contain fermenting and non-fermenting yeasts, lactic and acetic acid bacteria and moulds (Beech, 1993b). The origin of these micro-organisms is derived from both the apples and the harvesting procedures. An additional microflora is gained from the milling and pressing equipment, the degree of cleanliness of which varies the complexity of the microflora. The development of both yeast and bacteria in naturally fermented cider has been studied to some extent in French (Salih *et al*, 1988) and Spanish cider (Cabranes *et al.*, 1990) and to some extent, in English cider (Beech, 1972b; Beech and Carr, 1977; Beech, 1993b).

At the end of fermentation, commercially produced cider is racked off the lees and either fined immediately and left in storage or stored and fined later. When product is required, further clarification by centrifugation and/or filtration, using pulp filters, occurs. The bright cider may then be left for a further period in storage. In general, cider is stored for undefined periods between each clarification stage. Microbiological development during this disrupted storage has not been determined, nor have factors including aeration, agitation, removal of micro-organisms and particulate matter which affect cider microflora and subsequently maturation flavour.

In this work, observations of some effects of the three clarification procedures, racking, fining and filtration on the microbiological aspects of cider maturation were made. In particular, the diversity of type and numbers of micro-organisms present in cider at these different stages were examined. Representative micro-organisms were isolated from each cider sampled. Pure cultures of these isolates were obtained for investigations into effects of single micro-organisms on cider composition.

Lactic acid bacteria have an important role in Spanish and French cider making (Salih *et al.*, 1988; Cabranes *et* al., 1990) as well as in improving the flavour of wines (Lafon-Lafourcade *et al.*, 1983; Davis *et* al., 1985; Davis *et al.*, 1988; Pardo and Zuñiga, 1992), while acetic acid bacteria and non-*Saccharomyces* yeast are considered as spoilage micro-organisms.

Towards the end of fermentation, lactic acid bacteria grow more vigorously in the anaerobic conditions created by the yeasts, as low concentrations of carbon dioxide stimulate lactic acid bacteria (Carr, 1959; Leroi and Pidoux, 1993a). The bacteria utilise the remaining nutrients and recycle nitrogenous compounds released by the yeasts (Passmore and Haggett, 1973). A second fermentation is carried out by bacteria, universally known as malo-lactic fermentation (Beech, 1972b). It causes the conversion of malic acid to lactic acid and carbon dioxide. Malo-lactic fermentation is encouraged in wines for the purposes of deacidification, microbial stabilisation and/or modification of flavour and aroma (Kunkee, 1974; Lafon-Lafourcade and Ribereau-Gayon, 1984; Watson, 1986; Rodriguez *et al.*, 1990). It is thought that malo-lactic fermentation in cider imparts similar effects. In the commercial cider under investigation, malo-lactic fermentation was found to have already occurred before racking.

There is an increased recognition of the influence of malo-lactic fermentation on beverage quality, thus better control over the occurrence and outcome of this secondary fermentation are desirable. Selection of conditions to encourage the growth of natural microflora, induction of malo-lactic fermentation by inoculation with product already undergoing malo-lactic fermentation, induction by inoculation with pure lactic acid bacteria and lactic acid bacteria or enzyme immobilisation are methods under consideration for controlling malo-lactic fermentation (Davis *et al.*, 1985).

Pure culture induced malo-lactic fermentation is possibly the most desirable technique to employ. Thus, the ability of pure culture lactic acid bacteria isolated from storage cider to induce malo-lactic fermentation was studied as an initial step in the selection of suitable maturation micro-organisms.

7.2 EXPERIMENTAL

7.2.1 Isolation of Micro-Organisms from Cider at Various Stages of Storage

Samples from various storage vessels were aseptically collected in January and July (Table 7.1). A 10-fold serial dilution with 1/4 strength Ringer's solution, to 10^{-5} was performed on each of these samples. Spread plates of 0.1 ml aliquots of each dilution were made in triplicate onto WLNA, WLD, Raka Ray and Lysine Agars (Appendix I).

Table 7.1: Source of cider, stage of processing and season of isolation. Steel and concrete vats were epoxy resin lined.

VAT	VAT SPECIFICATIONS	STAGE	MONTH
Venus	Steel, 550, 000 gallons	Freshly Racked	July
Venus	Steel, 550, 000 gallons	Freshly Racked	January
Thatcher	Oak, 58,000 gallons	Racked and Settled	July
Lewis	Oak, 60,000 gallons	Racked and Settled	January
Redwing	Concrete, 102,000 gallons	Fined	July
Tedder	Concrete, 102,000 gallons	Fined	January
Swift	Concrete, 102,000 gallons	Fined	January
Kingfisher	Concrete, 102,000 gallons	Filtered	July
Geoffrey	Oak, 60,000 gallons	Filtered	January
Brockswood	Concrete, 102,000 gallons	Filtered Twice	July

After incubation, colonies that differed from one another on visual inspection were selected from each set of plates. These colonies were grown on their isolation medium, ensuring further selection of micro-organisms. When the cultures appeared to be pure, they were characterised and maintained (Section 3.1.2).

7.2.2 Macroscopic and Microscopic Morphology

The first step in identification of a pure culture was the examination and accurate description of the macroscopic morphology. The culture was then subjected to microscopic examination (Appendix II).

7.2.3 Qualitative Biochemical Tests used in the Characterisation of Bacteria

Cultures grown at 28°C were used for these tests. Young cultures were preferably used as old or poorly grown cultures might have given a false negative result. The standard tests used to characterise the bacteria were catalase, oxidase, Hugh and Leifson's oxidative/fermentative test and the production of acid and gas in Glucose Peptone Water, as detailed in Laboratoy Methods in Food and Drink Microbiology (Harrigan and McCance, 1976).

7.2.4 Malo-Lactic Activity of Lactic Acid Bacteria Isolates in Cider

Lactic acid bacteria from maintenance culture were streaked onto Raka Ray Agar and incubated at 28°C for seven days. Each isolate was inoculated into 20 ml filtered, laboratory fermented cider and incubated for five days at 25°C. This inoculum was then transferred to 500 ml of cider type A, in conical flasks. Carbon dioxide was permitted to escape via an airlock (containing distilled water) fitted to each conical flask, which also prevented oxygen penetration. Collection of samples from the sampling port was performed using a sterile syringe to draw off the requisite volume of cider. A sample was collected after inoculation and microbiological analysis was performed (Section 3.1.1) These flasks were then incubated at 25°C for four weeks. After four weeks, a sample was collected for microbiological counts and organic acid analysis (Section 3.4.2). A similar experiment was set up with cider type B, but only incubated for two weeks.

7.3 RESULTS

7.3.1 Isolation of Micro-Organisms from Cider at Various Stages of Storage

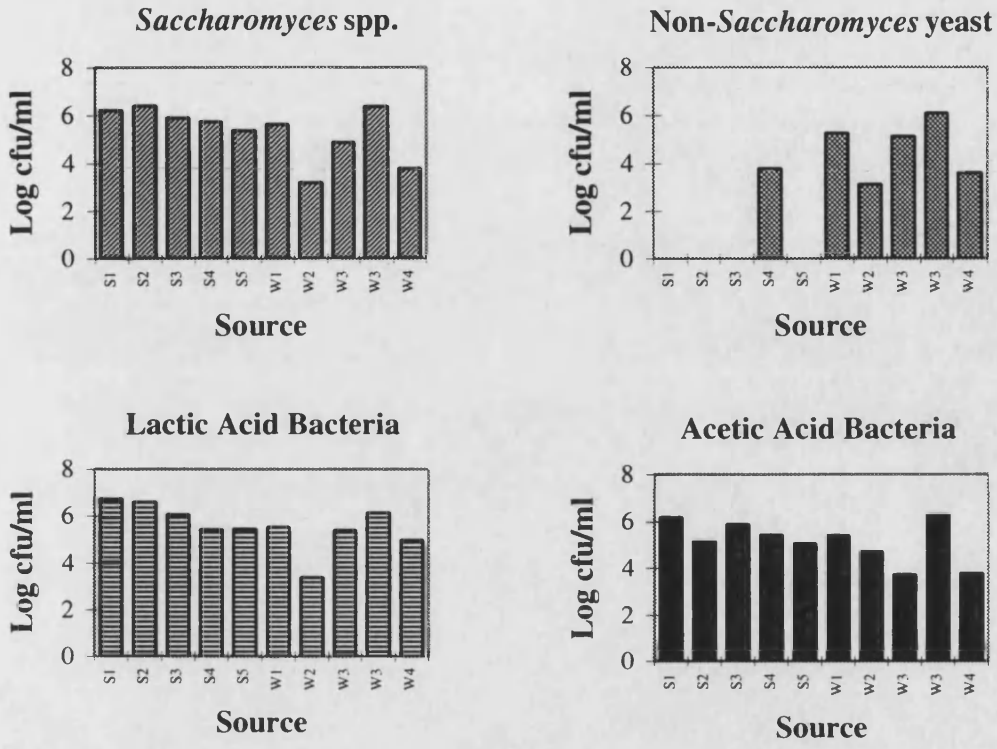

Figure 7.1 Distribution of micro-organisms in storage cider during winter (W) and Summer (S). Samples were collected from ciders at various stages of clarification: Newly racked from the lees (1), racked and settled (2), fined (3), filtered (4) and twice filtered (5).

The principle difference in population densities is between samples collected in the different seasons, namely summer and winter. It was observed that fewer micro-organisms were detected in cider during the winter compared with cider sampled in the summer, except in the case of non-*Saccharomyces* yeast, for which the opposite was the case.

There was an overall decrease in population densities throughout the clarification process in both summer and winter samples. The second W3 sample, however, possessed higher cell numbers for all four groups of micro-organisms. This sample was collected from a Woodpecker cider base, as opposed to a General Blend cider base. Woodpecker base is formulated with less juice and glucose than General Blend, but with an addition of molasses

(sucrose-based). Different formulations of apple juice base may therefore influence storage cider microflora.

Table 7.2 Number of Strains of Micro-Organisms in Each Group Isolated from Storage Ciders at Different Stages of Clarification

GROUP	STAGE OF CLARIFICATION				
	Racked	Racked/Settled	Fined	Filtered	Filtered x 2
Saccharomyces spp.	1	3	7	6	4
Non-*Saccharomyces* yeast	2	2	6	7	0
Lactic Acid Bacteria	12	24	17	10	22
Acetic Acid Bacteria	1	0	2	3	0

The majority of micro-organisms isolated from storage cider for purification and initial characterisation were lactic acid bacteria. The presence of several different strains of these bacteria throughout storage and clarification was detected. This contrasted with the limited number of different acetic acid bacteria detected.

A maximum of seven strains of *Saccharomyces* yeast was detected, in fined cider, while a maximum of seven possibly different strains of non-*Saccharomyces* were isolated from filtered cider. The number of strains of lactic acid bacteria isolated from racked and settled cider doubled from cider that had just been racked off the lees. A subsequent decrease in numbers of strains from 24 to a minimum of 10 in storage cider was observed after further clarification. Strain diversity returned after a second filtration stage.

7.3.2 Characterisation of Bacterial Isolates

Although yeast and bacteria had been isolated from the ten ciders detailed above, the bacteria were of greater interest at this stage. After purification, each bacterial isolate was characterised. Table 7.3 depicts the numbers and strains of bacteria isolated from storage cider.

Table 7.3: Bacterial genera found in storage cider

	Newly Racked	Racked	Fined	Filtered	Twice Filtered
Lactobacillus spp.	10	21	13	9	20
Leuconostoc spp.	1	3	3	0	0
Pediococcus spp.	0	1	1	1	2
Acetic Acid Bacteria	1	0	2	3	0
Gram-ve, fermentative rods	1	3	4	2	3
Zymomonas spp.	0	0	1	0	0
Others	0	1	0	1	4

Lactic acid bacteria were found to be the dominant bacteria in cider during storage, from freshly racked through to twice filtered. *Lactobacillus* spp. constituted 76% of the total number of bacterial strains isolated from racked cider, whereas *Leuconostoc* strains were present at a tenth of that level. *Pediococcus* spp. were not detected in freshly racked cider, although they were isolated from all other stages of storage. *Leuconostoc* spp. were not detected in filtered ciders, fined cider having been comprised of a maximum of 13% of the lactic acid bacteria population. The bacterial population of all ciders was primarily composed of strains of *Lactobacillus*, that is between 63% (filtered) and 86% (racked and settled).

7.3.3 Malo-Lactic Activity of Lactic Acid Bacteria Isolates in Cider

The ability of 62 of the 87 lactic acid bacteria isolated from storage cider to degrade malic acid was tested in order to commence selection of bacterial starters of high malo-lactic activity by inoculation of each strain into a sterile cider base.

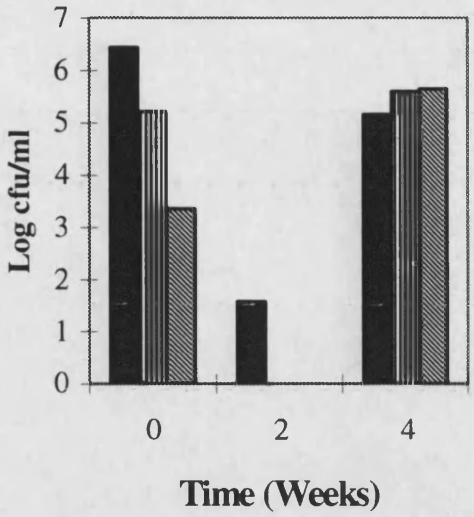

Figure 7.2: Average *Lactobacillus* spp. (■), *Leuconostoc* spp. (▩) and *Pediococcus* spp. (▧) cell counts in cider base A over a four week incubation at 25°C.

A decrease in lactic acid bacteria between time of inoculation and after two weeks incubation was evident, as illustrated in Figure 7.2. Both *Leuconostoc* and *Pediococcus* spp. were undetectable, having been inoculated to an average of 1.0×10^5 cfu/ml and 2.0×10^3 cfu/ml, respectively. This may be an effect of sampling error or genuine loss of viability. It was not possible to confirm which was the case. The initial inoculum level of each isolate was intended to be similar, but assessing numbers of cocci using a counting chamber was inaccurate, hence the variations in inocula. *Lactobacillus* strains were inoculated to the higher level of 2.8×10^6 cfu/ml and were still detectable, albeit in very low numbers, after two weeks. By the fourth week of incubation, the lactic acid bacteria populations had all increased to approximately 1.0×10^5 cfu/ml.

Figure 7.3 illustrates the mean organic acid profiles for each genus of lactic acid bacteria (Appendix V for data). The acetic acid concentration of cider A was noticeably elevated (between 0.3 to 0.6 g/l acetic acid would be acceptable). None of these isolates successfully induced malo-lactic fermentation within the four week incubation period.

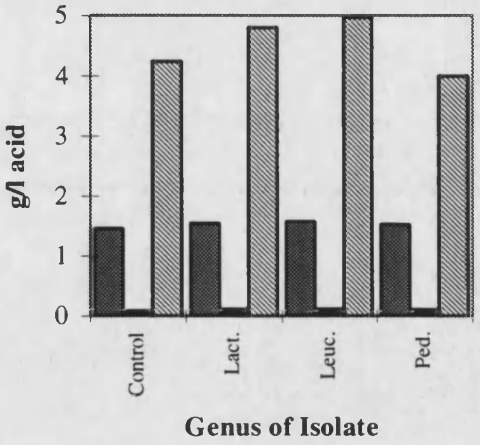

Figure 7.3: Mean concentration of malic (▩), lactic (■), acetic (▨) acid levels detected after inoculation of base cider A with individual lactic acid bacteria isolates after four weeks incubation at 25°C.

A second study with cider base B (with an acceptable level of acetic acid of 0.4 g/l) was performed with a second group of lactic acid bacteria isolates. The average results of this investigation are represented in Figure 7.4.

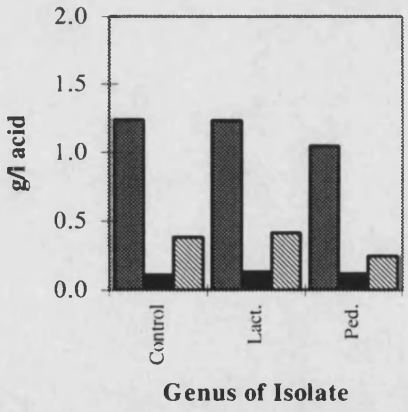

Figure 7.4: Average malic (■), lactic (■), acetic (▨) acid levels detected in base cider B after two weeks incubation at 25°C.

Again, the degradation of malic acid was not observed. The bacteria were inoculated into cider B at similar cell densities as those in cider A, with no apparent decline in population densities after two weeks incubation.

7.4 DISCUSSION

It was observed that the population densities of all micro-organisms, other than non-*Saccharomyces* yeast, was significantly higher in samples collected from cider conserved during summer months than during the winter months. An increased ambient temperature encourages microbial growth, (Beech and Carr, 1977; Joyeux *et al.*, 1984) while colder temperatures not only discourages microbial growth, but favours sedimentation, owing to less thermal movement, thus decreasing the number of micro-organisms in suspension.

The elevated non-*Saccharomyces* population observed in samples collected in winter is most probably due to the apple juice base used for fermentation. Whereas, cider stored during the winter months is primarily derived from fresh juice fermentations. Cider stored during the summer, prior to apple harvesting, is more commonly fermented from reconstituted apple juice concentrate. The process of concentration decreases the dissolved oxygen content of apple juice (Beech, 1993b) and renders the concentrate sterile of non-osmophilic micro-organisms (Beech, 1972a). It is also possible that ciders are conserved for longer during the winter (when demand is not so intense) allowing wild yeast populations to develop.

After racking, approximately 1.0×10^6 cfu/ml of *Saccharomyces* spp. were isolated. Levels of between 4.7 to 8.5×10^6 cfu/ml of *Saccharomyces* yeast had been reported in Spanish cider (Cabranes *et al.*, 1990), although in French cider a much reduced level of 5.8×10^4 cfu/ml was found (Salih *et al.*, 1988). At the end of storage, when Spanish and French ciders were ready for bottling, they contained levels of 3.0×10^4 and 2.3×10^3 cfu/ml respectively. At the analogous stage of storage, after the cider under observation had been filtered, levels between 10^4 and 10^5 cfu/ml were recorded. A combination of senescence and clarification procedures were considered to be responsible for the decline observed.

A similar scenario was observed with non-*Saccharomyces* yeast. At the beginning of storage, after racking, 1.0×10^5 cfu/ml of non-*Saccharomyces* spp. were detected in cider studied in winter and after pulp filter filtration, the population had decreased to 3.0×10^3 cfu/ml. In Spanish cider, *Kloeckera* sp. was isolated at levels between 2.5×10^5 and 4.5×10^5 cfu/ml after racking, but were undetectable at the end of storage, the length of which

was unclear (Cabranes *et al.*, 1990). In French cider, the initial population of non-*Saccharomyces* yeast was shown to be significantly lower than in Spanish cider (or the levels in cider in this investigation). At commencement of storage of French cider, 6.5 x 10^3 cfu/ml were isolated and had marginally reduced by the end of storage to 3.0 x 10^3 cfu/ml (Salih *et al.*, 1988).

In this study, acetic acid bacteria were observed to be present in levels up to 1.0 x 10^6 cfu/ml after racking, decreasing throughout storage to between 6.3 x 10^3 and 1.0 x 10^5 cfu/ml. Although acetic acid bacteria in Spanish cider is well documented (Salih and Suarez Diaz, 1990), the procedures by which Spanish cider is fermented is different from methods employed in the production of English commercial cider. After the malo-lactic fermentation of Spanish cider, acetic acid bacteria were reported at levels of 10^2 to 10^3 cfu/ml (Salih and Suarez Diaz, 1990).

Lactic acid bacteria in cider in this study were detected at levels between 5.0 x 10^6 and 5.0 x 10^7 cfu/ml after racking, gradually declining throughout storage and clarification to between 1.0 to 5.0 x 10^5 cfu/ml. Salih and his colleagues (1988) observed in French ciders that lactic acid bacteria populations increased from 6.8 x 10^6 cfu/ml to 1.1 x 10^7 cfu/ml. In a further study of French cider, between 1.0 x 10^5 and 1.2 x 10^7 cfu/ml of lactic acid bacteria were found throughout storage (Salih *et al.*, 1990).

The noticeable decline in population densities during storage is due partly to the clarification procedures employed (Beech and Carr, 1977) and a natural senescence of micro-organisms (Salih *et al.*, 1988; Cabranes *et al.*, 1990).

Comparisons between microbiological aspects of storage cider from Spain, France and England are complicated by dissimilar methods of production and processing. French and Spanish ciders were reported to show a decline in yeast populations throughout undisturbed storage. The cider in this study showed a similar yeast population decline, even when disturbed by clarification during storage, which would indicate that clarification processes have little or no effect on yeast populations, yet this conflicts with the purpose of clarification. Bacteria populations, however, were shown to remain constant in French and

Spanish ciders, but declined during storage in cider in this investigation. The implication is that clarification does affect bacterial numbers.

In order to obtain a clearer image of the effects of various clarification procedures on population densities and microbial diversity, as well as the differences occurring between the various cider recipes utilised, a more detailed study would be required. The move towards microfiltration immediately after racking will, however, render obsolete multiple clarification stages.

The increased number of strains isolated from fined and filtered ciders may be influenced by increased aeration as the cider is moved from one vessel to another allowing residual populations to establish themselves (Ribereau-Gayon, 1985; Salih and Suarez Diaz, 1990). Strain diversity, illustrated in table 7.4, is in agreement with previous observations that populations of lactic acid bacteria are the most varied in storage cider (Salih *et al.*, 1988). Vat microflora may also influence the number of strains isolated from cider transferred from one storage vat to another (refer to Chapter Six for details).

Lactic acid bacteria were the dominant micro-organisms in storage cider, which confirms the findings of Salih *et al.*, (1988) Cabranes *et al.*, (1990) on French and Spanish ciders. Lactic acid bacteria are well adapted for the harsh environment of cider, since they are anaerobic or microaerophilic micro-organisms capable of growing at a pH as low as 3.2 (Carr, 1959).

Davis *et al.*, (1986) found that some species of *Lactobacillus* were more tolerant of sulphur dioxide than many other lactic acid bacteria. These were presumed to be homofermentative bacteria, since heterofermentative rods are very sensitive to sulphur dioxide (Carr and Davies, 1972). In those ciders where the use of fairly high concentrations of sulphur dioxide is usual, the predominant bacteria were *Leuconostoc* spp. while *Lactobacillus* spp. predominated when low sulphur dioxide levels were present (Beech and Carr, 1977). Lactobacilli have the greatest tolerance of bound sulphur dioxide, leuconostocs intermediate and pediococci the least (Beech, 1993a). This is also indicated by the ratio of the three lactic acid bacteria genera isolated throughout storage.

Ratios during maturation, of the three different genera of lactic acid bacteria *Lactobacillus* spp. (86%), *Leuconostoc* spp. (8%) and *Pediococcus* spp. (6%) differ to those reported by Beech (1972b) who reported 57% *Leuconostoc* spp., 36% *Lactobacillus* spp. and 7% *Pediococcus* spp. in English ciders. These reported figures are, however, for cider throughout production, not just during storage, as in this situation. In addition, cider factories have their own, unique microflora (Beech, 1993a) and different processing techniques, which will cause variations in proportions of lactic acid bacteria in cider.

Acetic acid bacteria are strict aerobes, which survive during storage in wooden vats because of the penetration of oxygen into the wood (Joyeux *et al.*, 1984; Drysdale and Fleet, 1989). Passmore and Carr (1975) isolated seven strains of acetic acid bacteria from dry cider, which is comparable with the six strains isolated in this study. *Zymomonas* spp., tentatively identified in this study from fined cider, has been reported by Passmore and Carr (1975) and Salih and Suarez Diaz (1990). This micro-organism has been associated with cider sickness, which produces an aniseed-like flavour and a thick, creamy slime (Beech, 1972a).

The large, Gram negative, fermentative rods isolated from several ciders during storage were tentatively identified as belonging to the family Enterobacteriaceae. Goverd and his colleagues (1979) reported that coliforms formed part of the normal microflora of cider factories, especially in those using water flumes to wash and transport apples. These micro-organisms were found to decrease as fermentation commenced due to increasing ethanol concentrations and effects of yeast. Further identification and characterisation of these isolates would be required before any conclusions could be drawn as to their presence.

None of the 65 strains of lactic acid bacteria inoculated into cider was able to perform malo-lactic fermentation in the 28 day duration of the experiment. Pardo and Zuñiga (1992) found that only 2 of 45 strains of lactic acid bacteria isolated from wine performed malo-lactic fermentation when inoculated into wine, although all their strains could, to some degree ferment malic acid in a synthetic medium.

Cider, like wine, is a hostile environment for growth and metabolism of lactic acid bacteria. Low levels of nutrients and growth factors, low pH, presence of sulphur dioxide and high

concentrations of ethanol are all contributing factors to loss of bacterial viability after inoculation (Pardo and Zuñiga, 1992; Guzzo *et al.*, 1994). The cider in this study was indeed hostile, as growth factors and nutrients normally obtained from yeast autolysis (Passmore and Haggett, 1973) were unlikely to be present in sufficient quantities as extended contact on the lees did not occur, because the cider was filtered immediately after attenuation.

Cell death when bacteria are reinoculated into beer has been reported (Simpson and Fernandez, 1992), although the bacteria flourished when grown in beer and transferred from beer to beer. Although in this investigation, lactic acid bacteria readily grew in 20 ml volumes of cider, they did not continue to do so when transferred to a larger volume of cider. Loss of adaptation to cider owing to extended laboratory culture may partly be responsible for the responses observed in this investigation. The presence of sulphur dioxide is also reported to result in a rapid loss of cell viability, with growth recommencing at a later stage. Low pH levels increase the time taken for activity to commence (Calo *et al.*, 1991). Loss of cell viability was detected after two weeks incubation in cider A, although cell death is reported to occur within 24 hours (Guzzo *et al.*, 1994). The elevated level of acetic acid in cider A was the most probable case of this extended period of low cell viability. By 28 days, the bacterial populations were re-established, but the biomass was too low ($< 1.0 \times 10^6$ cfu/ml) to induce malo-lactic fermentation. In cider B, there was no apparent change in bacterial numbers throughout the incubation period. If an immediate loss of cell viability, followed by bacterial growth had occurred, it would have been overlooked, owing to infrequent sampling. Again, the resultant biomass was insufficient to induce malo-lactic fermentation and the incubation period was short. It is therefore possible that malic degradation would eventually have occurred if this investigation had been continued for several weeks.

7.5 SUMMARY

- Seasonal variations in storage cider microflora occurred. Populations were elevated in cider conserved during warmer months, most probably due to increased temperature.

- Yeast populations appear to decline naturally, irrespective of clarification procedures, whereas decreases in bacterial population densities were observed at each stage of clarification.

- Lactic acid bacteria exhibited the most strain diversity of all micro-organisms detected in storage ciders, with a possible 87 strains isolated.

- Inoculation of cider with lactic acid bacteria isolates resulted in initial cell death. This was followed by growth of the surviving, residual population. Malo-lactic fermentation was not induced as the critical inoculum level of 1.0×10^6 cfu/ml was not achieved. The hostile conditions of cider would delay growth and metabolic activities of the isolates, especially if they have reduced adaptation to sulphur dioxide, low pH and high ethanol concentration due to extended laboratory culture.

CHAPTER EIGHT

CONCLUSIONS

An intimate knowledge of flavour constituents, both volatile and non-volatile, their effects on cider flavour, their biochemistry and the effect of manufacturing variables is required for product optimisation. Pomology and fermentation has been and remains the main area of research in the cider industry. A review of literature concerned with flavour compounds in cider and microbiology of both ciders and wines was undertaken, which emphasised the lack of knowledge of cider maturation. The purpose of the research described in this thesis was to examine the influence of biochemical and microbiological factors on cider flavour development during storage.

Every cider maturation was determined to be unique, dependent upon many factors including apple juice used for fermentation, yeast strain, compounds synthesised during fermentation and the natural microflora of the cider and storage vats.

Ethyl lactate, 2-methyl-1-butanol and 2-phenyl ethanol are potential maturation marker compounds, as they were readily detected in cider and exhibited marked changes in concentration as maturation progressed.

It is recommended that volatile phenolic compounds, such as 4-ethyl phenol, 4-ethyl guaiacol and 4-ethyl catechol, and in particular their synthesis and influence on mature cider flavour, be the subject of further investigation.

Sterile fermented cider exhibited minimal flavour changes during storage, indicating a strong microbiological influence on maturation flavour development. In addition, non-microbial storage factors were determined as affecting cider flavour. Higher storage temperatures resulted in a cider with unpleasant, oxidised aromas, thus favouring lower storage temperatures. Storage of cider in the presence of oxygen indicated very profound and undesirable flavour changes, confirming the need to store cider in the absence of air. Post fermentation cider filtration affected flavour compounds in ciders derived from fresh

apple juice to a greater extent than those derived from apple juice concentrate fermentations.

Maturing cider was shown to possess a complex microflora, consisting of *Saccharomyces* and non-*Saccharomyces* yeast, acetic acid bacteria and lactic acid bacteria. These populations varied throughout the cider. Variations occurred throughout the duration of maturation as well as from season to season. Lactic acid bacteria, in particular *Leuconostoc* and/or *Pediococcus* spp., may have a significant role in cider maturation as they are capable of utilising residual carbohydrates, organic acids and phenolic acids. Cider that had undergone malo-lactic fermentation prior to maturation exhibited many more flavour changes during storage than cider that had undergone this deacidification process.

Wood storage vats were shown to possess a reservoir of micro-organisms, that could only be partially removed by cleaning procedures. These micro-organisms were demonstrated to have an influence on the total microbial population of cider and more importantly, the maturation flavour of cider stored in wood vats. These findings have several implications in the commercial production of cider. On one hand, as the characteristic spicy, complex cider flavour is replaced with a clean, crisp taste, the traditional cider character is lost. On the other hand, final product flavour would be more controlled, with the possible use of selected bacterial inocula to create a standard, desirable mature cider flavour.

Selection of a bacterial isolate to induce malo-lactic fermentation and cider maturation would have to consider the hostile nature of cider, which delays both bacterial growth and metabolic activities. In addition, the isolate would be required to create a desirable, reproducible flavour, preferably in as short a maturation period as possible.

REFERENCES

Abrishami, S.H., Tall, B.D., Bruursema, T.J., Epstein, P.S. and Shah, D.B. (1994) Bacterial adherence and viability on cutting board surfaces. *Journal of Food Safety* **14**, 153-172.

Ak, N.O., Oliver, D.O. and Kaspar, C.W. (1994) Cutting boards of plastic and wood contaminated experimentally with bacteria. *Journal of Food Protection* **57**, (1), 16-22.

Aldrich (1995) Flavors and Fragrances-International Edition, Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, 1-90.

Anon. (1994) A plastic approach: Interesting laboratory results on hygiene comparison of wood and plastic. *Kennedy's Confection* **1**, (13), 14.

Baumes, R., Cordonnier, R., Nitz, S. and Drawert, F. (1986) Identification and determination of volatile constituents in wines from different vine cultivars. *Journal of Science, Food and Agriculture* **37**, 927-943.

Beech, F.W. (1972a) Cider Making and Cider Research: A Review *Journal of the Institute of Brewing* **78**, 477-491.

Beech, F.W. (1972b) English Cidermaking. In *Progress in Industrial Microbiology: Volume 11.* ed. Hockenhull, D.J.D. pp. 133-214. Churchill Livingstone.

Beech F.W. (1993a) Cider Making and Cider Research. *Ferment* 259-270.

Beech, F.W. (1993b) Yeasts in Cider Making. In *The Yeasts: Volume 5.* ed. pp. 169-213.

Beech, F.W. and Carr, J.G. (1977) Cider and Perry. In *Economic Microbiology Volume 1: Alcoholic Beverages.* ed. Rose, A.H. pp. 139-314. Academic Press.

Benito de Cardenas, I.L., Peral de Portillo, M.C., Albizatti de Rivadeneira, M.C. and Oliver, G. (1991a) Effect of temperature on diacetyl and acetoin production by *Lactobacillus plantarum. Microbiologie - Aliments - Nutrition* **9**, 185-191.

Benito de Cardenas, I.L., Peral de Portillo, M.C., Albizatti de Rivadeneira, M.C., Medina, R. and Oliver, G. (1991b) Effect of citrate and glucose on growth and flavour compounds production by *Lactobacillus caseii* and *Lactobacillus plantarum. Microbiologie - Aliments - Nutrition* **9**, 229-237.

Bertuccioli, M. and Viani, R. (1976) Red wine aroma: Identification of headspace constituents. *Journal of Science, Food and Agriculture* **27**, 1035-1038.

Britz, T.J. and Tracey, R.P. (1990) The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos. Journal of Applied Bacteriology* **68**, 23-31.

Cabranes, C, Moreno, J and Mangas, J.J. (1990) Dynamics of yeast populations during cider fermentation in Asturian region of Spain. *Applied and Environmental Microbiology* **56**, (12), 3881-3884.

Calo, P., Cansado, J., Velázquez, J.B., Sieiro, E., Longo, E. and Villa, T.G. (1991) Effect of different physico-chemical conditions on malolactic fermentation of four *Lactobacillus Plantarum* wild strains isolated from wines of northwestern Spain. *Biotechnology Letters* **13**, (11), 781-786.

Carpentier, B. and Cerf, O. (1993) Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology* **75**, 499-511.

Carr, J.G. (1958) The vitamin requirements of lactic acid bacteria from ciders. *Antonie van Leeuwenhoek* **24**, 63-68.

Carr, J.G. (1959) Some special characteristics of the cider lactobacilli. *Journal of Applied Bacteriology* **22**, (3), 377-383.

Carr, J.G. and Davies, P.A. (1972) The ecology and classification of strains of *Lactobacillus collinoides* nov.spec.: A bacterium commonly found in fermenting apple juice. *Journal of Applied Bacteriology* **35**, 463-471.

Characklis, W.G., McFeters, G.A. and Marshall, K.C. (1989) Physiological ecology in biofilm systems. In *Biofilms* eds. W.G. Characklis and K.C. Marshall pp. 341-375 Wiley and Sons.

Chatonnet, P., Dubourdieu, D. and Boidron, J-N. (1991) Vinification et élevage des grands vins blancs secs en futs de chêne: le rôle des levures. *Revue des Oenologies* **61**, 53-55.

Chatonnet, P., Dubourdieu, D. and Boidron, J-N. (1992) Incidence of fermentation and ageing conditions of dry white wines in barrels on their composition in substances yielded by oak wood. *Sciences des Alimentes* **12**, 665-685.

Chatonnet, P., Dubourdieu, D., Boidron, J-N. and Lavigne, V. (1993) Synthesis of volatile phenols by *Saccharomyces cerevisiae* in wines *Journal of Science, Food and Agriculture* **62**, 191-202.

Clyne, J., Conner, J.M., Paterson, A. and Piggott, J.R. (1993) The effects of cask charring on Scotch Whisky maturation. *International Journal of Food Science and Technology* **28**, 69-81.

Cottrell, T.H.E. and McLellan, M.R. (1986) The effect of fermentation temperature on chemical and sensory characteristics of wines from seven white grape cultivars, grown in New York State. *American Journal of Enology and Viticulture* **37**, (3), 190-194.

Crapisi, A., Nuti, M.P., Zamorani, A. and Spettoli, P. (1987a) Improved stability of immobilized *Lactobacillus* sp. cells for the control of malolactic fermentation in wine. *American Journal of Enology and Viticulture* **38**, (4), 310-312.

Crapisi, A., Spettoli, P., Nuti, M.P. and Zamorani, A. (1987b) Comparative traits of *Lactobacillus brevis*, *Lact. fructivorans* and *Leuconostoc oenos* immobilized cells for the control of malo-lactic fermentation in wine. *Journal of Applied Bacteriology* **63**, 513-521.

Daudt, C.E. and Ough, C.S. (1973) Variations in some volatile acetate esters formed during grape juice fermentation. Effects of fermentation temperature, sulphur dioxide, yeast strain and grape variety *American Journal of Enology and Viticulture* **24**, (3), 130-135.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. and Fleet, G.H. (1985) Practical implications of malolactic fermentation: A Review. *American Journal of Enology and Viticulture* **36**, (4), 290-301.

Davis, C.R., Wibowo, D., Lee, T.H. and Fleet, G.H. (1986) Growth and metabolism of lactic acid bacteria during fermentation and conservation of some Australian wines. *Food Technology in Australia* **38**, (1), 35-40.

Davis, C.R., Wibowo, D., Fleet, G.H. and Lee, T.H. (1988) Properties of wine lactic acid bacteria: their potential enological significance. *American Journal of Enology and Viticulture* **9**, (2), 137-141.

Dicks, L.M.T. and van Vuuren, H.J.J. (1988) Identification and physiological characteristics of heterofermentative strains of *Lactobacillus* from South African red wines. *Journal of Applied Bacteriology* **64**, 505-513.

Drysdale, G.S. and Fleet, G.H. (1989) The effect of acetic acid upon the growth and metabolism of yeasts during the fermentation of grape juice. *Journal of Applied Bacteriology* **67**, 471-481.

Dubourieu, D. (1986) Wine Technology: Current trends. *Experientia* **42**, 914-921.

Durr, P. (1986) The flavour of cider. In *Developments in Food Science 3B: Food Flavours Part B The Flavour of Beverages* ed. Morton, I.D. and Macleod, A.J. pp. 85-97. Elsevier.

Etievant, P.X., Issanchou, S.N. and Bayonove, C.L. (1983) The flavour of Muscat wine: The sensory contribution of some volatile compounds. *Journal of Science, Food and Agriculture* **34**, 497-504.

Etievant, P.X. and Williams, A.A. (1984) Influence of storage on some sensory and physico-chemical characteristics of Beaujolais Nouveau wines. In *The Ninth Wine Subject Day: Current Technical Developments* October 22-23 Long Ashton Research Station, 1984, pp. 42-48.

Farris, G.A., Deiana, P., Budroni, M., Cabras, P., Spanedda, L. and Tuberoso, C. (1991) The transformation of organic acids in Vernaccia wine during flor formation *Journal of Fermentation and Bioengineering* **72**, (2), 138-140.

Fernandez , J.L. and Simpson, W.J. (1994) Cyclic growth of lactobacilli in beer *Journal of Applied Bacteriology* **77**, (6) 635-638.

Firme, M.P., Leitão, M.C. and San Romão, M.V. (1994) The metabolism of sugar and malic acid by *Leuconostoc oenos*: effect of malic acid, pH and aeration conditions. *Journal of Applied Bacteriology* **76**, 173-181.

Fleet, G.H., Lafon-Lafourcade, S. and Ribèreau-Gayon, P. (1984) Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology* **48**, (5), 1034-1038.

Fourcassie, P., Makaga-Kabinda-Massard, E., Belarbi, A and Maujean, A. (1992) Growth, D-glucose utilization and malolactic fermentation by *Leuconostoc oenos* strains in 18 media deficient in one amino acid. *Journal of Applied Bacteriology* **73**, 489-496.

Fredette, V. (1970) The scientific principles of wine making as illustrated with cider. *Journal of the Institute of Canadian Technology and Aliments* **3**, (4), A46-A49.

Gilberts, P., Evans, D.J. and Brown, M.R.W. (1993) Formation and dispersal of bacterial biofilms in vivo and in situ. *Journal of Applied Bacteriology Symposium Supplement* **74**, 67S-78S.

Gilliland. R.B. and Lacey, J.P. (1966) An *Acetobacter* lethal to yeasts in bottled beer *Journal of the Institute of Brewing* **72**, 291-303.

Goverd, K.A., Beech, F.W., Hobbs, R.P. and Shannon, R. (1979) The occurrence and survival of coliforms and salmonellas in apple juice and cider. *Journal of Applied Bacteriology* **46**, 521-530.

Guzzo, J., Cavin, J.F. and Divies, C. (1994) Induction of stress proteins in Leuconostoc oenos to perform direct inoculation of wine. *Biotechnology Letters* **16**, (11), 1189-1194

Harrigan, W.F. and McCance, M.E. (1976) In *Laboratoy Methods in Food and Drink Microbiology* Academic Press, London.

Henick-Kling, T. and Yun Hee Park (1993) Interactions of wine yeast and malolactic bacteria: Time of inoculation, sulphur dioxide and nutritional factors *Oregon State University* 209.

Henick-Kling, T., Acree, T., Gavitt, B.K., Krieger, S.A. and Laurent, M.H. (1993) Modification of wine flavour by malolactic fermentation *Oregon State University* 219-306.

Herraiz, T., Reglero, G., Martin-Alvarez, P.J., Herraiz, M. and Cabezudo, M.D. (1991) Identification of aroma components of Spanish `Verdejo' wine. *Journal of Science, Food and Agriculture* 103-116.

HMSO (1995) The Miscellaneous Food Additive Regulations 1995. 1995 Statutory Instrument 3187.

Hubert, C., Brunerie, P., Le Quere, J.M. and Drilleau, J.F. (1990) Volatile aroma compounds of ciders: Rapid extraction and analysis. *Sciences des Alimentes* **10**, 603-618.

Jarvis, B (1993) Cider (cyder; hard cider):Chemistry and microbiology of cidermaking. In *Encyclopediae of Food Science, Food Technology and Nutrition* ed Macrae, R., Robinson, R.K. and Sadler, M.J. pp. 984-989 Academic Press, London.

Jones, J.S., Sadler, G.D. and Nelson, P.E. (1986) Acetaldehyde and accelerated storage of wine: A new rapid method for analysis. *Journal of Food Science* **51**, (1), 229-230.

Joyeux, A., Lafon-Lafourcade, S. and Ribéreau-Gayon P. (1984) Evolution of acetic acid bacteria during fermentation and storage of wine *Applied and Environmental Microbiology* **48**, (1), 153-156.

Kaneko, T. and Yamamoto, Y. (1966) Killing of yeast by acetic acid bacteria *Report for the Research Laboratories, Kirin Brewery Company Limited* **9**, 37-50.

Killian, E. and Ough, C.S. (1979) Fermentation esters-formation and retention as affected by fermentation temperature. *American Journal of Enology and Viticulture* **30**, (4), 301-305.

King, S.W. and Beelman, R.B. (1986) Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. *American Journal of Enology and Viticulture* **37**, (1), 53-60.

Klingshirn, L.M., Liu, J.R. and Gallander, J.F. (1987) Higher alcohol formation in wines as related to the particle size profiles of juice insoluble solids. *American Journal of Enology and Viticulture* **38**, (3), 207-209.

Kösebalaban, F. and Özilgen, M. (1992) Kinetics of wine spoilage by acetic acid bacteria. *Journal of Chemistry, Technology and Biotechnology* **55**, 59-63.

Kunkee, R.E. (1967) Malo-lactic fermentation. *Advances in Applied Microbiology* **9**, 235-279.

Kunkee, R.E. (1974) Malo-Lactic Fermentation and Winemaking. In *Chemistry of Winemaking: Advances in Chemistry 137* Symposium 165th Meeting of the American Chemical Society April 12-13 1973. ed. Dinsmorr Webb, A. pp. 151-170. Washington D.C.: American Chemistry Society.

Lafon-Lafourcade, S., Carre, E., Louvard-Funel, A. and Ribereau-Gayon, P. (1983) Induction de la fermentation malolactique des vins par inoculation d'une biomasse industrielle congelée de *Leuconostoc oenos* après réactivation. *Connaissance Vigne Vin* **17**, (1), 55-71.

Lafon-Lafourcade, S. and Ribereau-Gayon, P. (1984) Developments in the Microbiology of Wine Production. In *Progress in Industrial Microbiology Volume 19: Modern Applications of Traditional Biotechnologies* ed. Bushell, M.E. pp. 1-46. Amsterdam, Oxford, New York, Tokyo: Elsevier.

Law, B.A. and Wigmore, A.S. (1982) Microbial proteinases as agents for accelerated cheese ripening. *Journal of the Society of Dairy Technology* **35**, (2), 75-76.

Lea, A.G.H and Timberlake, C.F. (1974) The phenolics of cider 1. Procyanidins. *Journal of Science, Food and Agriculture* **25**, 1537-1545.

Leguerinel, I., Cleret, J.J., Bourgeois, C. and Mafart, P. (1989) Yeast strains and kinetic aspects of formation of flavour compounds in cider. *Journal of the Institute of Brewing* **95**, 405-410.

Leguerinel, I., Cleret, J.J., Bourgeois, C. and Mafart, P. (1988) Yeast strain and the formation of flavour components in cider. *Journal of the Institute of Brewing* **94**, 391-395.

Leroi, F. and Pidoux, M. (1993a) Detection of interactions between yeasts and lactic acid bacteria isolated from sugary kefir grains. *Journal of Applied Bacteriology* **74**, 48-53.

Leroi, F. and Pidoux, M. (1993b) Characterization of interactions between *Lactobacillus hilgardii* and *Saccharomyces florentinus* isolated from sugary kefir grains. *Journal of Applied Bacteriology* **74**, 54-60.

Mahoney, S.P. (1993) Effects of sulphite on yeasts with special reference to intracellular buffering capacity. PhD Thesis, Bath, U.K.

Makanjuola, D.B., Tynom, A. and Springham, D.G. (1992) Some effects of lactic acid bacteria on laboratory-scale yeast fermentations. *Enzyme and Microbial Technology* **14**, pp. 350-357.

Megee, R.D., III., Drake, J.F., Fredrickson, A.G. and Tsuchiya, H.M. (1972) Studies in intermicrobial symbiosis. *Saccharomyces cerevisiae* and *Lactobacillus casei*. *Canadian Journal of Microbiology* **18**, (11), 1733-1742.

Michel, A., Bizeau, C. and Drilleau, J.F. (1988) Survey of the yeast flora found in cider factories located in the west of France. *Sciences des Aliments* **8**, 359-368.

Michel, A., Bizeau, C. and Drilleau, J.F. (1990) Relations metaboliques entre levures impliquees dans la fermentation du cidre. *Belgian Journal of Food Chemistry and Biotechnology* **45**, (3), 98-104.

Miller, J.C. and Miller, J.N. (1988) *Statistics for Analytical Chemistry* Second Edition.

Moir, M. (1992) The 1990 Laurence Bishop Silver Medal Lecture: The Desideratum for Flavour Control. *Journal of the Institute of Brewing* **98**, 215-220.

Momose, H., Iwano, K. and Tonoike, R. (1969) Studies on the aggregation of yeast caused by lactobacilli. *Journal of General Applied Microbiology* **15**, 19-26.

Moreno, J.J., Millàn, C., Ortega, J.M. and Medina, M. (1991) Analytical differentiation of wine fermentations using pure and mixed yeast cultures. *Journal of Industrial Microbiology*, 7, 181-190.

Nuraida L. and Owens, J.D. (1992) Acetoin production in *Leuconostoc mesenteroides* NCDO 518. *FEMS Microbiology Letters* **91**, 245-250.
Nykanen, L. (1986) Formation and occurrence of flavour compounds in wine and distilled alcoholic beverages. *American Journal of Enology and Viticulture* **37**, (1), 84-96.

Pardo, I. and Zuñiga, M. (1992) Lactic acid bacteria in Spanish red rosé and white musts and wines under cellar conditions. *Journal of Food Science* **57**, (2), 392-395.

Passmore, S.M. and Carr, J.G. (1975) The ecology of the acetic acid bacteria with particular reference to cider manufacture. *Journal of Applied Bacteriology* **38**, 151-158.

Passmore, S.M. and Haggett, B. (1973) The use of scanning electron microscopy to show confluent growth of a *Saccharomyces* and a *Leuconostoc* species. *Journal of Applied Bacteriology* **36**, 89-92.

Peddie, H.A.B. (1990) Ester formation in brewery fermentations. *Journal of the Institute of Brewing* **96**, 327-331.

Piggott, J.R., Conner, J.M., Paterson, A. and Clyne, J. (1993) Effects on Scotch Whisky composition and flavour of maturation in oak casks with varying histories. *International Journal of Food Science and Technology* **28**, 303-318.

Pollard, A., Kieser, M.E., Stevens, P.M. and Tucknott, O.G. (1965) Fusel oils in cider and perries. *Journal of Science, Food and Agriculture* **16**, 384-389.

Pollard, A., Kieser, M.E. and Beech, F.W. (1966) Factors influencing the flavour of cider: The effect of fermentation treatments on fusel oil production. *Journal of Applied Bacteriology* **29**, (2), 253-259.

Radler, F. (1992) The second fermentation in wine: Malic acid degradation, discovery application, consequences, and control. *Biologia Oggi* **VI**, (1-2), 3-7.

Rapp, A. and Mandery, H. (1986) Wine Aroma. *Experientia* **42**, 873-884.

Ribereau-Gayon, P. (1985) New developments in wine microbiology. *American Journal of Enology and Viticulture* **36**, (1), 1-10.

Rodriguez, S.B., Amberg, E., Thornton, R.J. and McLellan, M.R. (1990) Malolactic fermentation in Chardonnay: growth and sensory effects of commercial strains of *Leuconostoc oenos*. *Journal of Applied Bacteriology* **68**, 139-144.

Rosini, G. (1984) Assessment of dominance of added yeast in wine fermentation and origin of Saccharomyces cerevisiae in wine making. *Journal of General Applied Microbiology* **30**, 249-256.

Saha, R.B., Sondag, R.J. and Middlekauff, J.E. (1974) An improved medium for the selective culturing of lactic acid bacteria. *Proceedings for the American Society of Brewing Chemists, 9th Congress*, 1974.

Ribereau-Gayon, P. (1985) New developments in wine microbiology. *American Journal of Enology and Viticulture* **36**, (1), 1-10.

Rodriguez, S.B., Amberg, E., Thornton, R.J. and McLellan, M.R. (1990) Malolactic fermentation in Chardonnay: growth and sensory effects of commercial strains of *Leuconostoc oenos*. *Journal of Applied Bacteriology* **68**, 139-144.

Rosini, G. (1984) Assessment of dominance of added yeast in wine fermentation and origin of Saccharomyces cerevisiae in wine making. *Journal of General Applied Microbiology* **30**, 249-256.

Saha, R.B., Sondag, R.J. and Middlekauff, J.E. (1974) An improved medium for the selective culturing of lactic acid bacteria. *Proceedings for the American Society of Brewing Chemists, 9th Congress*, 1974.

Sakuma, S., Hayashi, S. and Kobayashi, K. (1990) Analytical methods for beer flavour control. *Journal of American Society of Brewing Chemists* **49**, 1-3

Salih, A.G., Drilleau, J.-F., Cavin, F.F., Divies, C. and Bourgeois, C.M. (1988) A Survey of Microbiological Aspects of Cider Making. *Journal of the Institute of Brewing* **94**, 5-8.

Salih, A.G., Drilleau, J.-F., Divies, C., Lenzi, P., Calvez, J., Le Quéré, J.M., Bohuon, G. and Gestin, P. (1987) Facteurs contribuant au contrôle de la transformation malolactique dans les cidres. *Sciences des Aliments* **7**, 205-221.

Salih, A.G., Le Quéré, J.-M., Drilleau, J.-F. and Moreno Fernandez, J. (1990) Lactic acid bacteria and malolactic fermentation in the manufacture of Spanish cider. *Journal of the Institute of Brewing* **96**, 369-372.

Salih, A.G. and Suarez Diaz, A. (1990) Les bactérias acétiques dans le cidre de la province des Asturies (Espagne). *Sciences des Aliments* **10**, 75-88.

Schreier, P. (1979) Flavour composition of wines: A Review. *CRC Critical Reviews in Food Science and Nutrition* **12**, 59-111.

Schreier, P., Drawert, F. and Schmid, M. (1978) Changes in the composition of neutral volatile components during the production of apple brandy. *Journal of Science, Food and Agriculture* **29**, 728-736.

Shimazu, Y., Uehara, M. and Watanabe, M. (1985) Transformation of citric acid to acetic acid, acetoin and diacetyl by wine making lactic acid bacteria. *Agricultural and Biological Chemistry* **49**, (7), 2147-2157.

Simpson, W.J. and Fernandez, J.L. (1992) Selection of beer spoilage lactic acid bacteria and induction of their ability to grow in beer. *Letters in Applied Microbiology* **14**, 13-16.

Suomalainen, H. and Lehtonen, M. (1979) The production of aroma compounds by yeast. *Journal of the Institute of Brewing* **85**, 149-156.

Torrent, J., Batalla, G., Marco, L., Sendros, A. and Vidal, J.F. (1989) Improvement of process control by new HPLC techniques to maintain aroma volatiles beer composition. European Brewery Convention Proceeding of the 22nd Congress 1989 757-764.

Tracey, R.P. and van Rooyen, T.J. (1988) Utilization of glucose, fructose and malic acid by malolactic bacteria: Effect of ethanol and formation of mannitol and volatile acids. *Journal of Applied Bacteriology* **65**, 113-118.

Tsau, J.-L., Guffanti, A.A. and Montville, T.J. (1992) Conversion of pyruvate to acetoin helps to maintain pH homeostasis in *Lactobacillus plantarum*. *Applied and Environmental Microbiology* **58**, (3), 891-894.

Velázquez, J.B., Calo, P., Longo, E., Cansado, J., Sieiro, C. and Villa, T.G. (1991) Effect of L-Malate, D-Glucose and L-Lactate on malolactic fermentation and growth of *Lactobacillus plantarum* and *Lactobacillus curvatus* wild strains isolated from wine. *Journal of Fermentation and Bioengineering* **71**, (5), 363-366.

Voilley, A., Lamer, C., Dubois, P. and Feuillat, M. (1990) Influence of macromolecules and treatments on the behaviour of aroma compounds in a model wine. *Journal of Agriculture and Food Chemistry* **38**, 248-251.

Walker, M.D. and Simpson, W.J. (1993) Production of volatile sulphur compounds by ale and lager brewing strains of *Saccharomyces cerevisiae*. *Letters in Applied Microbiology* **16**, 40-43.

Watson, B. (1986) Malolactic Fermentation: A Review of Current Practices, Problems, and Research at OSU. In *The Wine Advisory Board Research Report- Oregon Wines*. pp. 1-5. Oregon Department of Agriculture: The Wine Advisory Board.

Whiting, GC (1976) Organic acid metabolism of yeasts during fermentation of alcoholic beverages - A review. *Journal of the Institute of Brewing* **82**, 84-92.

Whittenbury, R. (1963) The use of soft agar in the study of conditions affecting the utilization of fermentation substrates by lactic acid bacteria. *Journal of General Microbiology* **32**, 375-384.

Wibowo, D., Fleet, G.H., Lee, T.H. and Eschenbruch, R.E. (1988) Factors affecting the induction of malolactic fermentation in red wines with *Leuconostoc oenos*. *Journal of Applied Bacteriology* **64**, 421-428.

Williams, A.A. (1974) Flavour research and the cider industry. *Journal of the Institute of Brewing* **80**, 455-470.

Williams, A.A. (1982) Recent developments in the field of wine flavour research. *Journal of the Institute of Brewing* **88**, 43-53.

Williams, A.A. (1989) Post fermentative changes in wines with particular reference to the volatile flavour components of sherry and port. In Proceedings of the International Symposium on *The Aromatic Substances in Grapes and Wines* June 25-27 Italy 1989 Ed. A. Scienza and G. Versini 201-222.

Williams A.A. and Tucknott, O.G. (1978) the volatile aroma components of fermented ciders: Minor neutral components from the fermentation of Sweet Coppin apple juice. *Journal of Science, Food and Agriculture* **29**, 381-397.

Yurchenko, L.A., Vecher, A.S., Prokazov, G.F., Vasilkevich, S.I. and Romanovets, E.S. (1974) Effects of storage conditions on fermented apple juice. *Prikladnaya Biokhimiya i Mikrobiologiya* **10**, (6), 903-907.

Zúñiga, M., Pardo, I. and Ferrer, S. (1993) An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. *International Journal of Food Microbiology* **18**, 37-42.

APPENDIX I: MEDIA

W.L. Nutrient Agar

W.L. Nutrient Medium (Unipath Ltd., U.K.)	75 g
Distilled/deionised water	1000 ml

75 g of W.L. Nutrient Medium was suspended in 1 l distilled water and boiled, stirring constantly. The medium was sterilised by autoclaving at 121°C for 15 minutes.

W.L. Differential Agar

W.L. Nutrient Agar powder (Unipath Ltd., U.K.)	75 g
Cycloheximide, 0.1% stock solution	10 ml
Distilled/deionised water	1000 ml

75 g W.L. Nutrient Agar powder was dissolved in 1 l distilled water by bringing it to the boil, stirring constantly. 10 ml cycloheximide stock solution were added and mixed well. The medium was sterilised by autoclaving at 121°C for 15 minutes.

Raka Ray Agar

Raka Ray medium powder (Unipath Ltd.,	77.1 g
Tween 80 (BDH Ltd., U.K.)	10 ml
Phenyl ethanol (BDH Ltd., U.K.)	3 g
Cycloheximide 0.1% stock solution	7 ml
Distilled/deionised water	1000 ml

77.1 g Raka Ray agar powder was suspended in 1 l distilled water with 10 ml Tween 80 (a dispersant) and 7 ml 0.1% cycloheximide stock solution. The medium was sterilised by autoclaving at 121°C for 15 minutes. The medium is allowed to cool to 50°C and 3 g phenyl ethanol was added aseptically.

Lysine Agar

Lysine Medium powder (Unipath Ltd., U.K.)	66 g
Distilled/deionised water	1000 ml
Potassium Lactate, 50% (BDH Ltd., U.K.)	10 ml
Lactic Acid, 10% (BDH Ltd., U.K.)	1 ml

66 g of Lysine Medium powder was suspended in 1 l distilled water containing 10 ml potassium lactate 50% solution. The mixture was boiled, stirring continuously to prevent superheating and to ensure complete dissolution of the agar. The medium was cooled to 50°C and 1 ml sterile lactic acid 10% solution was added, adjusting the pH to approximately 5.0.

The medium was dispensed into Petri dishes and used immediately. Storage prior to use was not recommended due to vitamin degradation.

Cycloheximide Stock Solution (0.1%w/v)

0.1 g of cycloheximide (BDH Ltd., U.K.) was weighed out into a medical flat and 100 ml distilled water is added. The solution is sterilised by autoclaving at 121°C for 15 minutes. This poisonous stock solution was stored at 4°C.

Ringer's Solution

1 tablet of 1/4 strength Ringer's was dissolved in 500 ml distilled water. The solution was dispensed into clean, glass Universals in volumes of 9 ml and then sterilised by autoclaving at 121°C for 15 minutes.

Basal Broth

Lab Lemco powder (Unipath Ltd., U.K.)	5 g
Bacteriological Peptone (Unipath Ltd., U.K.)	5 g
Yeast Extract (Unipath Ltd., U.K.)	5 g
D-Glucose (BDH Ltd., U.K.)	5 g
Tween 80 (BDH Ltd., U.K.)	0.5 g
Distilled/deionised water	1000 ml

All the ingredients are dissolved in the water and boiled. The medium is sterilised by autoclaving at 121°C for 15 minutes. Dispense aseptically, into sterile, 7 ml Bijous.

MYGP Agar

Malt Extract (Unipath Ltd., U.K.)	3 g
Yeast Extract (Unipath Ltd., U.K.)	3 g
Mycological Peptone (Unipath Ltd., U.K.)	5 g
D-Glucose (BDH Ltd., U.K.)	10 g
Agar (Unipath Ltd., U.K.)	20 g
Distilled/deionised water	1000 ml

All the ingredients were dissolved in the water and boiled. The medium was sterilised by autoclaving at 121°C for 15 minutes. 7 ml aliquots were aseptically dispensed into sterile, 28 ml Universals and allowed to set at an angle.

Glucose Peptone Water - Fermentation Acid and Gas Detection

D-Glucose (BDH Ltd., U.K.)	10 g
Bacteriological Peptone (Unipath Ltd., U.K.)	10 g
Andrade's Base (Unipath Ltd., U.K.)	0.1 g
Distilled/deionised water	1000 ml

The ingredients were dissolved in the water and 3 ml aliquots were dispensed into Bijous containing an inverted Durham tube. The containers and their contents were sterilised by autoclaving at 121°C for 15 minutes.

Hugh and Leifson Medium - Oxidative/Fermentative Test

Bacteriological Peptone (Unipath Ltd., U.K.)	2 g
NaCl (BDH Ltd., U.K.)	5 g
K2HPO4 (BDH Ltd., U.K.)	0.3 g
Bromothymol Blue (BDH Ltd., U.K.)	0.03 g
D-Glucose (BDH Ltd., U.K.)	10 g
Agar (Unipath Ltd., U.K.)	3 g
Distilled water	1000 ml

All the ingredients were dissolved in boiling water and dispensed in 10 ml aliquots into clean test tubes. The test tubes and their contents were sterilised by autoclaving at 121°C for 15 minutes.

APPENDIX II : MORPHOLOGY

MACROSCOPIC

AMOUNT OF GROWTH (Diameter in mm)

Scant

Moderate

Abundant

CONSISTENCY

Butyrous	butter like growth
Viscous	follows needle when withdrawn
Membranous	
Brittle	dry, friable

FORM

Punctiform	under 1 mm
Circular	
Filamentous	long, irregular interwoven threads
Rhizoid	irregular, branched
Irregular	

ELEVATION

Effuse/diffuse	very thin, spreading
Flat	
Raised	
Convex	
Pulvinate	
Umbonate	

MARGIN

Entire	smooth, no notches
Undulate	wavy, shallow indentations
Erose	irregularly notched
Filamentous	irregular, interwoven edges
Curled	parallel chains in wavy strands

SURFACE

Smooth	
Contoured	irregular, undulating
Radiate	ridges radiating from centre
Concentric	rings with common centre
Rugose	wrinkled

OPTICAL CHARACTERISTICS

Pigmentation	none or specific colour, fluorescent
Opaque	not permitting light to pass through
Translucent	some light passes through but not enough to permit visibility
Opalescent	opal like appearance, milky iridescence
Iridescent	exhibiting changing rainbow colours in reflected light
Dull	not glossy
Glistening	glossy

MICROSCOPIC

SHAPE

Bacillus	rod, cylinder
Coccus	spherical, ovoid
Curved, cylindrical	Spirillum, Spirochete, Vibrio

STAINING CHARACTERISTICS

Gram reaction	positive or negative

GROUPING

Bacillus	single, pair, chain
Coccus	single
Micrococcus	single
Diplococcus	pair, with flattened sides
Streptococcus	chain
Staphylococcus	clusters of spheres with geometric arrangement
Tetrad	cells in flat plates of four
Sarcina	cubical packets of eight

APPENDIX III - CIDER FLAVOUR DESCRIPTIVES

AROMATIC	Alcoholic	**ASTRINGENT**	Drying
	Estery (pear drops)		Bitter
	Fruity		
	Floral		
	Vegetative		
ACIDIC	Sour	**SOLVENTY**	Acetone
	Sharp		Plastic
	Acetic/Vinegar		Medicinal
	Sweaty		Petrol
OXIDISED	Acetaldehyde	**FATTY**	Soapy
	Stale		Cheesy
	Musty		Goaty
	Papery		Buttery
			Waxy
			Oily
SULPHURY	Hydrogen Sulphide	**BODY**	Watery
	Garlic		Thin
	Burnt Rubber		Characterless
	Onion		Thick
	Cooked Vegetable		Balanced
	Yeasty		Warming

APPENDIX IV

a) Levels (mg/l) Of Volatile Flavour Compounds In Ciders: Effect Of Temperature #1

Temperature	5°C					15°C					25°C				
Week	2	5	8	11	16	2	5	8	11	16	2	5	8	11	16
ethyl-2-methyl butyrate	0.6	0.6	0.6	0.5	0.5	0.6	0.4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.4
hexanal	20.9	20.6	20.7	16.0	14.2	21.8	32.0	15.4	15.5	31.1	22.7	20.6	16.0	14.5	14.4
iso amyl acetate	0.5	0.7	0.5	0.5	0.5	0.5	0.5	0.5	2.6	0.5	0.6	0.5	2.7	0.6	0.7
2-methyl-1-butanol	0.0	0.0	0.0	0.0	0.0	0.0	53.7	0.0	0.3	0.3	0.0	0.0	0.3	0.0	0.0
ethyl hexanoate	1.1	1.0	1.1	0.9	0.9	1.0	0.9	0.9	0.9	0.9	0.9	0.9	0.4	0.9	0.8
hexyl acetate	1.3	0.0	0.0	0.0	0.0	0.6	0.0	0.0	1.3	1.3	0.0	0.0	0.2	0.0	0.0
octanal	1.3	10.1	1.3	1.3	1.3	1.3	1.4	1.3	1.3	1.3	1.3	7.7	0.6	2.5	2.0
ethyl lactate	37.4	46.6	34.4	28.7	26.7	34.5	26.6	27.0	25.2	28.5	31.7	37.1	29.9	31.1	34.4
hexanol	4.1	3.4	3.8	3.4	3.3	3.9	3.1	3.2	3.0	3.2	3.6	3.4	1.9	3.5	3.5
nonanal	1.5	1.5	1.5	1.4	1.4	1.5	1.4	1.4	1.4	1.4	1.5	1.5	0.4	1.5	1.5
ethyl octanoate	1.3	6.6	1.3	1.3	1.2	1.3	1.2	1.2	1.1	1.1	1.3	1.2	0.3	1.1	1.1
undecanal	2.7	2.7	3.0	2.8	2.8	2.7	2.7	2.7	2.8	2.8	2.8	2.7	0.4	2.9	2.9
ethyl decanoate	1.0	1.0	1.0	1.0	0.9	1.0	0.0	0.9	0.9	0.9	1.0	1.0	0.2	3.9	4.7
ethyl benzoate	1.3	2.4	2.5	2.3	2.2	1.8	1.9	1.2	2.0	2.1	2.4	2.5	1.3	1.2	1.2
diethyl succinate	6.1	4.3	5.7	4.5	4.5	5.4	3.4	4.2	3.0	3.2	4.7	4.7	3.5	3.9	3.8
phenylethyl acetate	4.9	4.1	4.4	3.7	3.4	5.3	4.0	3.5	2.8	2.9	5.7	5.0	2.4	3.4	3.1
ethyl dodecanoate	2.5	2.3	4.0	3.5	3.6	3.1	2.8	3.5	3.1	3.2	3.7	3.8	3.8	3.8	3.9
2-phenyl ethanol	2.0	1.8	20.3	16.0	15.6	2.0	25.6	1.8	14.4	14.7	1.9	19.8	14.3	1.9	16.5
heptanoic acid	3.3	3.2	5.7	5.2	5.1	4.3	4.2	5.2	4.6	4.6	5.3	5.7	5.6	5.4	5.6
octanoic acid	4.5	4.4	5.2	5.0	4.9	4.8	4.6	4.7	4.5	4.5	5.1	4.9	1.1	4.6	4.7
4-ethyl phenol	6.5	3.4	4.8	0.4	5.1	5.3	1.1	0.3	2.4	3.0	4.1	3.3	5.4	5.8	6.6

b) Levels (mg/l) Of Volatile Flavour Compounds In Ciders: Effect Of Temperature #2

Temperature	5°C			15°C			25°C		
Week	0	4	11	0	4	11	0	4	11
ethyl-2-methyl butyrate	1.3	1.4	1.5	1.1	1.3	1.3	1.0	1.2	1.0
hexanal	16.5	14.9	16.9	15.5	15.8	14.1	15.0	14.9	14.7
iso amyl acetate	0.7	0.9	0.7	0.7	0.8	0.9	0.7	0.7	0.6
2-methyl-1-butanol	37.6	26.4	33.2	38.4	28.9	33.3	39.3	36.0	33.5
ethyl hexanoate	0.9	0.9	0.9	0.9	0.9	1.0	0.9	0.9	0.9
hexyl acetate	1.3	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
octanal	1.4	1.5	1.4	1.4	1.5	1.6	1.5	1.5	1.4
ethyl lactate	3.3	3.4	3.3	3.6	4.0	7.5	3.8	5.6	7.7
hexanol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
nonanal	1.5	1.6	1.5	1.6	1.6	1.8	1.6	1.5	1.4
ethyl octanoate	2.8	2.9	2.8	2.8	2.8	3.0	2.7	2.8	2.8
undecanal	1.1	1.2	1.2	1.1	1.2	1.2	1.1	1.1	1.2
ethyl decanoate	1.4	1.4	1.4	1.8	1.5	1.7	1.6	1.5	1.3
ethyl benzoate	0.7	0.7	0.7	0.7	0.7	0.9	0.7	0.8	0.9
diethyl succinate	5.2	5.4	4.6	5.2	5.4	6.3	5.1	4.5	3.8
phenylethyl acetate	3.4	4.2	3.5	3.4	4.0	3.4	3.4	4.0	3.4
ethyl dodecanoate	8.4	9.1	8.3	9.2	9.9	9.9	10.0	9.5	11.5
2-phenyl ethanol	4.2	4.9	4.4	4.2	4.7	5.8	4.1	4.8	5.1
heptanoic acid	4.9	5.6	5.0	5.2	5.6	6.6	5.2	5.8	5.7
octanoic acid	0.1	0.0	0.0	0.1	0.1	0.0	0.1	0.2	0.2
4-ethyl phenol	0.2	0.2	0.4	0.3	0.4	0.2	0.2	0.7	0.5

c) Levels (mg/l) Of Volatile Flavour Compounds In Ciders: Effect Of Filtration #1

Filtration	Unfiltered					Post-Microfiltered					Post-Ultrafiltered				
Week	2	5	8	11	16	2	5	8	11	16	2	5	8	11	16
ethyl-2-methyl butyrate	1.8	0.6	0.7	0.5	0.4	1.1	0.9	1.4	0.6	0.5	0.4	1.9	0.4	0.4	0.5
hexanal	47.6	14.7	15.1	13.6	20.8	14.2	13.4	15.3	16.3	0.6	15.6	18.7	12.3	14.5	14.0
iso amyl acetate	1.1	0.4	0.5	0.6	3.4	0.6	0.7	0.7	0.6	2.1	0.4	0.7	0.4	0.5	0.8
2-methyl-1-butanol	0.3	29.6	28.0	27.4	0.3	0.3	0.0	0.4	0.4	0.4	0.0	0.0	27.1	27.9	0.4
ethyl hexanoate	1.3	0.8	0.8	0.8	1.1	1.0	1.0	1.4	0.8	0.8	0.0	0.8	0.8	0.8	0.8
hexyl acetate	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.4	0.0	0.0	0.0
octanal	1.8	1.4	1.4	1.5	2.1	1.6	1.4	2.7	1.6	1.9	1.7	2.0	2.2	1.6	1.7
ethyl lactate	1.4	37.2	30.4	35.5	59.4	7.0	34.4	27.0	34.1	42.0	8.1	21.1	8.9	10.6	15.0
hexanol	32.1	2.9	3.3	2.9	5.5	4.6	4.0	6.8	3.1	4.6	3.4	3.1	3.4	3.4	4.0
nonanal	1.7	1.4	1.4	1.4	1.5	1.5	1.5	1.6	1.4	1.7	1.4	1.4	1.4	1.4	1.4
ethyl octanoate	21.5	1.0	1.0	1.0	1.3	5.0	6.3	1.0	1.0	1.2	1.0	2.0	1.0	1.0	1.0
undecanal	3.1	2.9	2.7	2.9	3.2	2.9	2.8	3.3	2.7	2.7	2.8	2.7	2.7	2.7	2.7
ethyl decanoate	1.1	4.3	1.0	6.9	18.9	1.0	1.0	1.0	0.0	1.0	1.0	0.9	1.0	0.0	0.9
ethyl benzoate	1.3	1.3	1.2	1.2	1.3	1.3	1.3	1.3	1.3	1.3	1.2	1.3	2.5	2.6	3.1
diethyl succinate	0.8	4.9	5.7	5.3	11.8	8.7	7.2	6.1	5.3	2.3	0.9	0.9	1.1	1.1	1.3
phenylethyl acetate	1.2	2.9	1.2	3.1	1.3	1.3	3.9	1.3	3.5	1.8	3.1	3.3	1.2	3.0	0.0
ethyl dodecanoate	6.6	3.0	3.5	3.2	4.7	4.4	3.6	3.5	3.4	4.1	3.1	3.8	3.4	3.3	3.5
2-phenyl ethanol	22.1	13.5	13.5	12.9	18.7	13.5	12.7	13.5	14.2	16.7	12.6	10.2	12.1	12.5	13.5
heptanoic acid	8.9	4.6	5.2	5.1	6.5	6.5	5.4	5.2	5.1	6.9	4.7	5.7	5.2	5.1	5.1
octanoic acid	6.3	4.3	4.5	4.4	5.0	5.0	4.6	4.5	4.5	5.0	4.3	4.8	4.3	4.3	4.5
4-ethyl phenol	3.6	0.4	0.8	1.0	3.5	1.1	0.8	1.2	1.6	1.8	1.4	2.0	0.4	0.2	3.1

d) Levels (mg/l) Of Volatile Flavour Compounds In Ciders: Effect Of Filtration #2

Filtration	Unfiltered			Microfiltered			Ultrafiltered		
Week	0	4	11	0	4	11	0	4	11
ethyl-2-methyl butyrate	0.9	1.2	1.2	1.0	1.0	1.3	1.8	1.5	0.3
hexanal	11.5	15.2	14.7	14.1	12.3	15.0	21.5	17.5	26.5
iso amyl acetate	0.7	0.7	0.7	0.7	0.6	0.7	0.6	0.5	0.6
2-methyl-1-butanol	26.0	29.3	27.9	29.3	26.4	27.7	35.5	30.6	31.9
ethyl hexanoate	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.7	0.9
hexyl acetate	1.3	1.4	1.4	1.3	1.4	1.4	1.3	1.3	1.4
octanal	1.4	1.5	1.5	1.5	1.4	1.6	1.7	1.5	1.6
ethyl lactate	3.1	4.1	4.8	3.9	3.1	4.3	5.3	3.6	18.6
hexanol	0.0	0.0	0.0	0.0	0.0	0.0	1.8	1.8	1.9
nonanal	0.0	0.0	0.0	0.0	0.0	0.0	1.4	1.4	1.4
ethyl octanoate	1.6	1.6	1.6	1.6	1.4	1.4	1.6	1.6	1.6
undecanal	2.8	2.9	2.8	2.9	2.8	2.8	3.2	3.0	3.1
ethyl decanoate	1.2	1.1	1.2	1.1	1.0	1.0	1.0	1.0	1.0
ethyl benzoate	1.3	1.5	1.5	1.5	1.4	1.5	1.8	1.6	1.3
diethyl succinate	0.7	0.8	0.8	0.7	0.7	0.8	0.7	0.7	2.1
phenylethyl acetate	4.6	5.3	4.9	6.5	4.1	4.5	1.2	6.0	1.2
ethyl dodecanoate	4.0	4.0	3.9	4.3	3.6	4.0	6.1	4.9	5.7
2-phenyl ethanol	8.8	10.4	9.8	11.1	7.8	9.1	13.5	10.8	9.7
heptanoic acid	4.9	4.7	4.6	4.9	4.6	4.8	6.5	5.8	6.1
octanoic acid	5.4	5.7	5.6	6.5	5.2	5.6	7.6	6.1	7.6
4-ethyl phenol	0.2	0.1	0.1	0.6	0.4	0.1	0.9	0.6	0.4

e) Levels (mg/l) Of Volatile Flavour Compounds In Ciders: Effect Of Aeration #1

Treatment	Carbon Dioxide			Oxygen		
Week	0	9	16	0	9	16
ethyl-2-methyl butyrate	0.5	0.5	0.5	0.6	0.5	0.7
hexanal	18.9	25.3	23.3	18.6	18.0	27.2
iso amyl acetate	0.6	0.5	0.5	0.7	0.5	0.5
2-methyl-1-butanol	0.0	43.6	41.6	0.0	0.3	48.8
ethyl hexanoate	0.9	0.9	0.9	1.1	0.9	1.0
hexyl acetate	0.0	0.0	0.0	1.3	1.3	0.0
octanal	1.3	1.3	1.8	1.4	5.2	0.0
ethyl lactate	29.3	28.4	27.5	33.9	26.2	37.0
hexanol	3.3	3.4	3.1	4.4	4.5	4.0
nonanal	1.5	1.5	1.5	1.6	1.5	1.5
ethyl octanoate	1.3	1.2	1.2	1.6	1.2	1.3
undecanal	2.7	0.0	2.7	2.7	3.2	0.0
ethyl decanoate	1.0	1.0	1.0	1.1	1.0	1.0
ethyl benzoate	1.3	2.1	2.0	1.3	1.2	2.3
diethyl succinate	6.8	3.9	4.0	7.9	3.1	5.2
phenylethyl acetate	1.2	4.4	4.6	1.3	3.7	4.0
ethyl dodecanoate	2.3	2.2	2.2	2.7	2.2	2.3
2-phenyl ethanol	19.8	20.7	20.0	2.0	1.9	24.5
heptanoic acid	2.8	3.2	3.3	3.3	3.3	3.2
octanoic acid	4.6	4.1	4.1	5.1	4.1	4.5
4-ethyl phenol	2.5	2.5	2.6	3.1	3.0	3.4

f) Levels (mg/l) Of Volatile Flavour Compounds In Ciders: Effect Of Aeration #2

Treatment	Oxygen		Carbon Dioxide	
Week	**0**	**5**	**0**	**5**
ethyl-2-methyl butyrate	3.3	3.1	3.1	0.4
hexanal	19.6	18.6	19.4	3.3
iso amyl acetate	0.5	0.5	0.5	0.4
2-methyl-1-butanol	28.6	27.4	28.7	5.9
ethyl hexanoate	1.4	1.1	1.4	1.4
hexyl acetate	1.6	1.6	1.6	2.2
octanal	1.4	1.5	1.4	1.3
ethyl lactate	5.1	5.6	5.0	6.9
hexanol	1.8	0.0	1.8	0.0
nonanal	1.4	0.0	1.4	0.0
ethyl octanoate	1.1	1.1	1.1	1.0
undecanal	2.8	2.8	2.8	2.8
ethyl decanoate	0.5	0.0	1.0	2.4
ethyl benzoate	1.3	1.2	1.3	1.3
diethyl succinate	0.8	0.7	0.8	0.8
phenylethyl acetate	5.0	5.5	4.7	4.3
ethyl dodecanoate	4.0	3.9	3.9	3.4
2-phenyl ethanol	6.6	6.9	6.7	6.7
heptanoic acid	5.3	5.0	5.2	4.9
octanoic acid	5.3	5.1	5.5	4.3
4-ethyl phenol	0.0	0.0	0.0	0.0

g) Levels (mg/l) Of Volatile Flavour Compounds In Ciders: Effect Of Agitation #1

Treatment	Static		Circulating	
Week	0	3	0	3
ethyl-2-methyl butyrate	2.0	6.0	2.0	1.0
hexanal	16.0	48.0	17.0	16.0
iso amyl acetate	0.0	8.0	0.0	3.0
2-methyl-1-butanol	34.0	0.0	39.0	0.0
ethyl hexanoate	0.0	59.0	0.0	0.0
hexyl acetate	2.0	1.0	2.0	1.0
octanal	3.0	39.0	3.0	20.0
ethyl lactate	6.0	3.0	7.0	2.0
hexanol	2.0	11.0	2.0	2.0
nonanal	0.0	7.0	0.0	4.0
ethyl octanoate	0.0	1.0	0.0	36.0
undecanal	3.0	3.0	3.0	3.0
ethyl decanoate	0.0	1.0	0.0	1.0
ethyl benzoate	1.0	1.0	1.0	1.0
diethyl succinate	1.0	5.0	1.0	14.0
phenylethyl acetate	2.0	1.0	3.0	5.0
ethyl dodecanoate	2.0	2.0	2.0	2.0
2-phenyl ethanol	12.0	18.0	12.0	11.0
heptanoic acid	0.0	3.0	0.0	3.0
octanoic acid	4.0	5.0	4.0	4.0
4-ethyl phenol	1.0	9.0	1.0	2.0

APPENDIX V

Chapter Seven - Induction Of Malo-Lactic Fermentation By Lactic Acid Bacteria Isolates

Sample	mg/l organic acid Malic	Lactic	Acetic	Citric	Succinic	Log cfu/ml at Week 5
Cider Base	1215	111	387	43	702	0.00
Control 1	1241	110	383	46	856	0.00
Control 2	1459	92	4237	46	235	0.00
Lactobacillus spp.						
002a	1685	149	3162	0	349	0.71
009a	1565	84	7160	0	327	6.80
009a	1386	142	532	0	1019	0.00
011	1316	127	432	0	789	3.10
014	1656	136	2055	0	334	0.00
020	1425	142	455	0	860	3.10
021	1726	125	4878	0	336	5.66
025	1164	114	531	0	874	0.00
027a	1144	123	491	0	877	5.40
028	1063	119	487	0	858	5.08
030	1272	147	537	0	966	4.93
031	1634	118	4430	0	123	5.65
032	1116	109	536	0	861	0.00
046	1339	137	570	0	991	0.00
047	1513	112	4423	0	108	0.00
049	1531	133	4635	0	106	5.40
059	1262	138	610	0	945	4.88
060a	1326	101	3969	0	0	5.40
063	1176	119	575	0	881	4.00
066	1330	134	134	0	971	0.00
069a	1679	113	0	0	0	5.54
070	1172	139	267	0	0	3.10
076	1419	109	303	0	0	5.33
082a	1202	103	0	0	0	1.40
083	1365	106	6426	0	0	5.70
087a	1023	113	326	0	0	3.42
087b	1428	88	0	0	0	6.63
088	1183	128	580	0	0	4.10
090a	1359	153	326	0	0	6.00
090b	1115	159	234	0	0	5.01
091a	1523	106	0	0	0	5.70

Sample	Malic	mg/l organic acid Lactic	Acetic	Citric	Succinic	Log cfu/ml at Week 5
094	1255	120	303	0	0	2.30
096	1551	98	267	0	0	5.88
102a	1113	127	292	96	0	3.70
102b	1502	119	104	101	0	5.10
105	1095	120	909	107	0	3.88
109	1516	157	207	0	0	6.00
110	1223	144	288	0	0	3.35
114	1528	118	0	68	0	5.53
115	1132	217	280	67	0	4.78
118	1111	126	271	67	0	0.00
121	1631	107	6576	54	311	6.00
124	1304	118	333	73	973	0.00
132	1298	132	323	71	943	4.10
138	1343	124	304	70	954	5.04
143b	1510	141	4961	76	309	5.40
145	1249	157	325	71	944	4.54
146	1267	128	325	70	926	4.10
148b	1291	119	323	74	956	2.30
149	1316	123	328	74	978	0.00
151	1492	156	760	57	1144	4.93
152a	1294	121	488	62	997	2.68
155	1207	125	462	59	939	4.60
158	1241	121	381	46	877	4.72
160	1226	178	411	47	894	5.28
Leuconostoc spp.						
142	1570	142	4828	77	298	5.40
101	1611	109	6661	0	0	5.40
023	1469	102	4224	0	96	5.40
024	1583	111	4457	0	111	5.88
010	1569	112	4674	0	309	6.35
Pediococcus spp.						
069b	1047	117	250	0	0	4.68
157	1509	108	3886	62	279	4.30
015	1550	111	4088	0	298	7.00